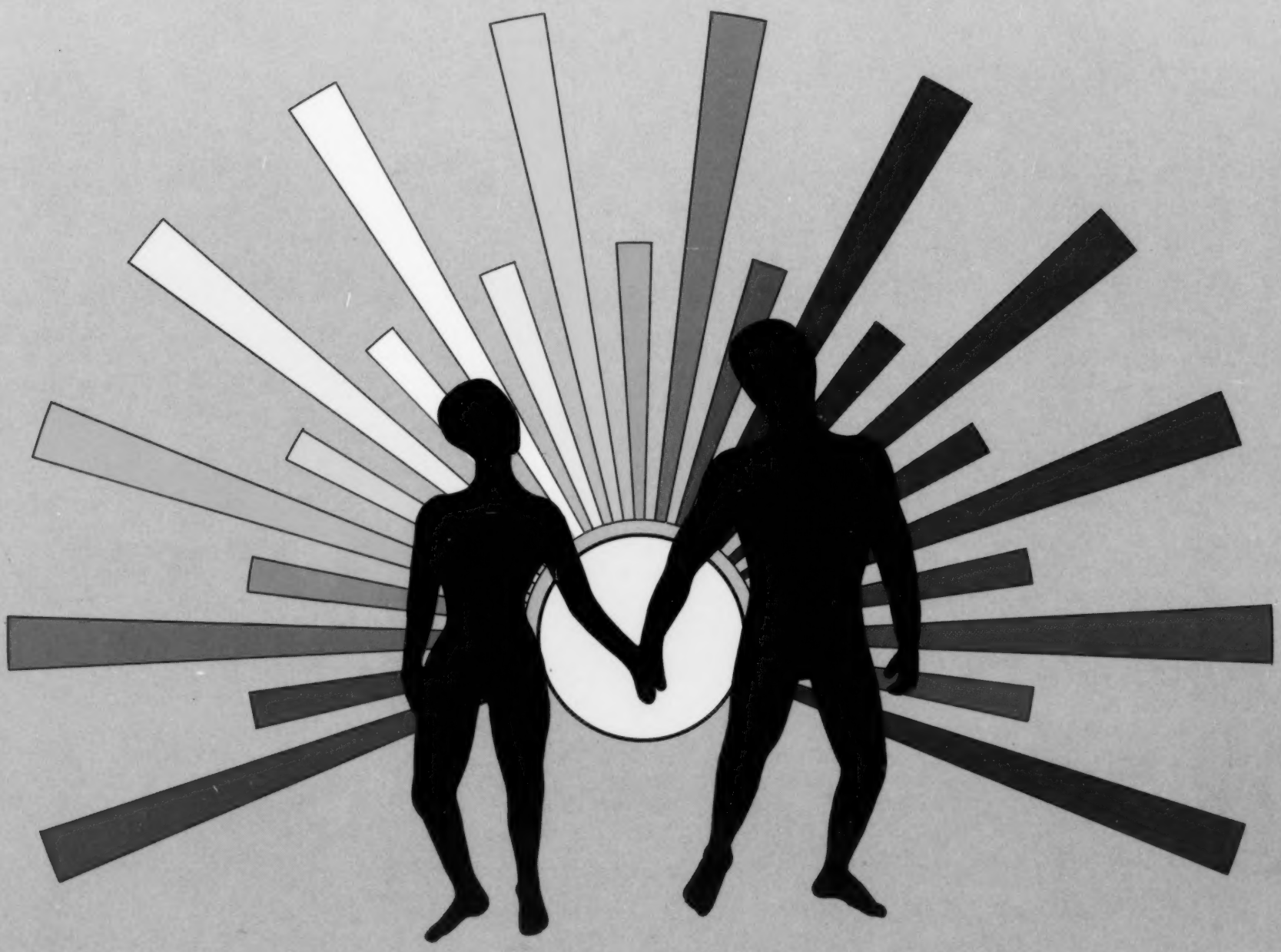
Volume 94 Nos 1/2 May 1st, 1990

ISSN 0048-9697
published in 36 issues per year

the Science of the Total Environment

An International Journal for Scientific Research into the Environment and its Relationship with Man



SPECIAL ISSUE:
CHEMICAL CONTAMINANTS AND FISH TUMORS
Edited by C.D. Metcalfe

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the Science of the Total Environment

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volume 94 (1990)

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PRINTED IN THE NETHERLANDS

SPECIAL ISSUE

CHEMICAL CONTAMINANTS AND FISH TUMORS

Edited by

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CONTENTS

Preface

Chemical Contaminants and Fish Tumors

Epizootiology of neoplasms in bony fish from North America.
J.C. Harshbarger and J.B. Clark (*Washington, D.C., USA*) 1

Overview of studies on liver carcinogenesis in English sole from Puget Sound; Evidence for a xenobiotic chemical etiology I: Pathology and epizootiology.
M.S. Myers, J.T. Landahl, M.M. Krahn, L.L. Johnson and B.B. McCain (*Seattle, Washington, USA*) 33

Overview of studies on liver carcinogenesis in English sole from Puget Sound; Evidence for a xenobiotic chemical etiology II: Biochemical studies.
J.E. Stein, W.L. Reichert, M. Nishimoto and U. Varanasi (*Seattle, Washington, USA*) 51

Relationship between liver tumors and age in brown bullhead populations from two Lake Erie tributaries.
P.C. Baumann (*Columbus, Ohio, USA*), J.C. Harshbarger (*Washington, D.C., USA*) and K.J. Hartman (*Columbus, Ohio, USA*) 71

^{32}P-Postlabeling detection of DNA adducts in fish from chemically contaminated waterways.
A.E. Maccubbin, J.J. Black (*Buffalo, N.Y., USA*) and B.P. Dunn (*Vancouver, B.C., Canada*) 89

Pathogenesis of skin and liver neoplasms in white suckers from industrially polluted areas in Lake Ontario.
M.A. Hayes (*Guelph, Ontario, Canada*), I.R. Smith (*Rexdale, Ontario,Canada*), T.H. Rushmore (*Toronto, Ontario, Canada*), T.L. Crane, C. Thorn, T.E. Kocal and H.W. Ferguson (*Guelph, Ontario, Canada*) 105

Carcinogenic and genotoxic activity of extracts from contaminated sediments in western Lake Ontario.
C.D. Metcalfe, G.C. Balch (*Peterborough, Ontario, Canada*),V.W. Cairns, J.D. Fitzsimons (*Burlington, Ontario, Canada*), B.P. Dunn (*Vancouver, B.C., Canada*) 125

A possible correlation between environmental chemicals and pigment cell neoplasia in fish.
Naohide Kinae, Mitsuko Yamashita, Isao Tomita (*Shizuoka, Japan*),Ikuo Kimura, Hiroji Ishida (*Nagoya, Japan*), Hidemi Kumai and Genji Nakamura (*Wakayama, Japan*) 143

Carcinogenic effects of some polycyclic aromatic hydrocarbons on the Japanese medaka and guppy in waterborne exposures.
W.E. Hawkins, W.W. Walker, R.M. Overstreet, J.S. Lytle and T.F. Lytle (*Ocean Springs, Mississippi, USA*) 155

PREFACE

Almost thirty years ago, reports began to appear in the scientific literature speculating on the role that environmental pollution might play in the development of cancer-like lesions in fish. Up until relatively recently, the evidence for a cause-and-effect relationship between environmental contaminants and fish tumors has been based, rather tenuously, upon observations of fish tumors in areas impacted by environmental pollution. Conversely, there have been data which negate the concept of a chemical etiology for tumors in wild fish populations. Surveys of fish inhabiting the highly polluted Sava, Rhine, and Elbe Rivers in Europe, and the industrialized Port Phillip Bay in Australia show that fish tumors are not common at these sites. There is strong epidemiological, biochemical, and ultrastructural evidence that oncogenic viruses or parasitic infections are the causative agents for a variety of neoplasms in fish, including some skin papillomas, gonadal tumors, and lymphatic tumors.

Beginning in about 1974, fish tumor surveys were initiated in the industrialized southern portion of Puget Sound, in the state of Washington, USA; an area in which liver tumors were observed in large numbers of English sole. These survey data, plus more recent data generated by an on-going multidisciplinary research program, have provided the most complete documentation supporting an association between exposure of fish to chemical contaminants and the development of neoplasms. Two reviews of the most recent advances in the Puget Sound study are included in this issue. In recent years, other multi-disciplinary research programs have been initiated to examine fish tumor epizootics in several areas of North America, as well as Japan. This issue provides a summary of some of the most recent data gathered in some of these studies.

What kinds of tumors are caused by chemical contaminants, as opposed to the tumors induced by the oncogenic activity of viruses and parasites? Can the carcinogenic chemicals causing these tumors be identified? Do these effects upon fish health have ramifications for the protection of human health? These questions are addressed in this special issue on "Chemical Contaminants and Fish Tumors".

The general objective of this issue is to review the most recent data on the role of chemical contaminants in the development of tumors in fish. However, the specific goals are to define the types of fish neoplasms which could be associated with a chemical etiology, and to identify classes of compounds which may be responsible for chemical carcinogenesis in fish. There is an emphasis upon multi-disciplinary studies that integrate epidemiological, pathological, analytical, and experimental carcinogenesis data on specific populations of tumor-bearing fish.

Without detracting from the contents of this special issue, it would be useful to highlight some of the major conclusions that can be drawn from it. Polycyclic aromatic hydrocarbons (PAHs) are almost certainly involved in the development of tumors in feral fish populations inhabiting contaminated marine and freshwater ecosystems. This assumption is supported by a considerable body of epidemiological data, in association with PAH analysis of

sediments, experimental carcinogenesis studies, and data on levels of aromatic metabolites and aromatic chemical-DNA adducts in fish. Considerable work is needed to identify the specific PAH compounds involved in tumor development, or whether related nitrogen and sulfur heterocyclics are also carcinogenic.

Despite the weight of information available on PAH-related fish carcinogenesis, it is important not to rule out other environmental mixtures as etiological agents. This is illustrated in these proceedings by data collected in Japan, where skin neoplasms in marine fish are related to exposure to kraft pulp mill effluents containing, among other constituents, genotoxic chlorinated and carbonyl compounds. Work is also required to determine the significance of simultaneous exposures to other environmental contaminants, such as PCBs, with the potential to modify tumorigenesis in feral fish.

Liver tumors are the specific neoplasms in feral fish which are most strongly associated with a chemical etiology; specifically, with exposure to PAH compounds. Liver neoplasms arising from the proliferation of malignant hepatocytes are almost certainly induced by exposure to chemical contaminants, but the etiology of liver tumors of biliary origin is less clear. More work is required to define the pathogenesis of cholangiolar neoplasms in fish, and in particular, the role of putative disease-chemical interactions in liver neoplasia. While considerable attention has been focused on hepatic tumors, neoplasms of epithelial origin should not be ignored as indicators of exposure to chemicals. The development of malignancy among these neoplasms may be related to chronic inflammation induced by exposure to chemicals. These papers indicate that chemically-induced carcinogenesis in fish resembles mammalian carcinogenesis, in that it is a "multi-factorial" disease, requiring a precise sequence of cellular events progressing towards malignancy.

As indicated in several of the papers, high incidences of tumors are found almost exclusively among populations of bottom-dwelling fish; presumably because of their close association with contaminated sediments and their relatively sedentary habits. Progress is being made towards defining why, even among bottom-dwelling species, some feral fish are more sensitive to environmental carcinogens than others. The data presented in this issue indicate that interspecific variations in metabolic detoxification pathways may explain differences in tumor incidence among closely related flatfish species. However, other variables, such as interspecific differences in DNA repair and tumor immuno-surveillance in fish have not been adequately studied. Analysis of levels of chemical-DNA adducts in fish may be an excellent tool for studying interspecific variations in tumor incidence.

A link was established between the development of fish tumors and the ecological health of a fish population. Specifically, data indicating selective mortality of older individuals from a tumor-bearing population of brown bullheads indicates that fish neoplasia can have a negative impact upon fish health and the management of fish stocks. It is often difficult to establish that human health is being threatened in areas where fish tumors have been observed in large numbers. In many cases, tumor-bearing fish inhabit areas in which the water resource is not being used for drinking water or recreation. Consumption of flesh from even tumor-bearing fish may not constitute a significant health hazard because of the relatively rapid metabolism and excretion of carcinogens, such as PAHs, from the organism.

However, there seems to be no doubt that the appearance of neoplasms in fish, particularly liver tumors, is an indicator of environmental degradation within a geographic region. We are often unable to precisely define the contaminated region using fish tumor

data because of the migratory habits of some species. The appearance of some types of fish tumors should be regarded as a signal that remedial or abatement action is required. It would seem self-evident that efforts to reverse the degradation of marine and freshwater systems can be justified on the basis of human aesthetics, protection of our fisheries resources, and the general maintenance of environmental quality, rather than only to safeguard human health.

ACKNOWLEDGEMENTS

A symposium on "Chemical Contaminants and Fish Tumors" leading to the publication of this special issue was sponsored by the Ontario Ministry of the Environment. Funds for editing and publication costs were provided from the operating funds of the Canadian "Annual Aquatic Toxicity Workshop", and from an operating grant to CDM from the Natural Sciences and Engineering Research Council of Canada. Thanks to Gary Westlake, David Rokosh, and Art Niimi for their suggestions and help. Many thanks to the authors of the manuscripts, and to the reviewers for their prompt attention to the manuscripts. A special thanks is extended to Debbie Mills for her help in preparing the manuscripts for publication. Gord Balch, Nakos Kotsanis, Mark Nanni, and Chris Williams proof-read the manuscripts.

The Science of the Total Environment, 94 (1990) 1–32
Elsevier

EPIZOOTIOLOGY OF NEOPLASMS IN BONY FISH OF NORTH AMERICA

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ABSTRACT

Over the past 25 years, there have been an increasing number of fish tumor epizootics recognized in discrete geographical areas in North America. This is consistent with an association between tumor development in fish and exposure to waterborne contaminants as a result of increased industrialization. Reports of fish tumor epizootics from the literature, plus acquisitions at the Registry of Tumors in Lower Animals, Smithsonian Institution originate from 41 geographical regions in North America. Epizootics of hemic, neural, connective tissue, and gonial neoplasms seem unrelated to environmental pollution, but epizootics of hepatic and epidermal neoplasms appear to be caused or strongly influenced by environmental pollution. The 14 species with epizootic liver tumors are all bottom feeding fish, and tumor-bearing fish are generally located in areas where chemical contaminants are concentrated. Epidermal neoplasms are also found in fish near polluted areas, but a direct cause-and-effect link with chemical carcinogens is not clear.

INTRODUCTION

Reports of neoplasms in ectothermic animals began appearing in the scientific literature during the last half of the 19th century, and by 1900, tumors had been described in reptiles, amphibians, bony fish, and bivalve molluscs. By 1963, the cytologic, phyletic, and geographic distribution of fish neoplasms had been considerably expanded, but despite extensive studies by Johnstone, Takahashi, Lucke, Schlumberger, and others, only a few epizootic neoplasms had been reported in fish from the wild. These epizootics comprised three basic types: epidermal papilloma, peripheral nerve sheath cell tumors, and lymphoma.

Epidermal papillomas were described in the European smelt, *Osmerus eperlanus,* from the Baltic Sea (Breslauer, 1916), in the European eel, *Anguilla anguilla*, from the coastal waters of Holland, Denmark, and Germany (Christiansen and Jensen, 1950), in the brown bullhead, *Ictalurus nebulosus*, from the Delaware and Schuylkill Rivers near Philadelphia, Pennsylvania (Lucke and Schlumberger, 1941a), and in the white croaker, *Genyonemus lineatus*, from the southern California coast (Russell and Kotin, 1957). Nerve sheath cell tumors were reported in the gray snapper, *Lutjanus griseus*, from the Florida keys (Lucke, 1938; Lucke, 1942), and peripheral nerve sheath cell tumors were reported in the goldfish,

Carassius auratus, from an isolated lagoon (Schlumberger, 1952). Lymphoma was described in the northern pike, *Esox lucius*, from Ireland (Mulcahy, 1963).

In 1964, liver neoplasms were first reported in wild fish; in white sucker, *Catostomus commersoni*, and brown bullhead from Deep Creek Lake, Maryland, and it was suggested for the first time that environmental chemical pollutants might be the cause (Dawe et al., 1964). During the subsequent 25 years, several dozen wild fish tumor epizootics were identified in North America, a preponderance of which originated in the liver tissue of bottom feeders clustered in areas where pollutants are concentrated. These epizootics are documented in this study, and are mapped to aid in an understanding of the interrelationships between tumor type, fish species, and geographic distribution.

METHODS

Epizootics were determined from the accession records of the Registry of Tumors in Lower Animals (RTLA) as compiled by Harshbarger (1965-1989) and combined with reports in the literature. At least three cases originating in a specific cell lineage in a defined geographic location were required to be considered an epizootic. These locations (sites) were numerically indicated on maps of North America, along with a code for the fish species and general tumor type.

Pertinent references, RTLA accession numbers and anecdotal information, including other types of tumors found in fish in this location, are given in the text. Specific diagnoses used in the text generally conform to the terminology used by the author or the RTLA at the time of study, but in some cases terminology has been updated to enhance clarity.

The text is subdivided into geographic regions and listed numerically according to the numbered sites on the maps. Epizootics are first listed by species and then by tumor type. In Table 1, there is a listing of the site number, site, species, and epizootic to provide an overview of the data. Thus, by using the maps, text, and table in combination, the three basic parameters (location, species and tumor type) are readily cross-referenced.

For conciseness, the following abbreviations were used in the text, table, and on the maps:

Tumors

cn = cholangiocytic neoplasm
ds = dermal sarcoma
ep = epidermal papilloma
fs = fibrosarcoma
gt = gonadal tumor
hn = hepatocytic neoplasm
lm = leiomyoma
ls = lymphosarcoma
ns = nerve sheath neoplasms, e.g., schwannoma, neurofibroma, neurilemoma
on = odontogenic neoplasm
pc = pigment cell neoplasm, e.g., chromatoblastoma, chromatophoroma, melanoma
pn = pancreatic neoplasm
sc = squamous carcinoma

Table 1: Listing of fish tumor epizootics in North America.

SITE #	FISH	EPIZOOTIC	LOCATION	NUMBER OF RTLA CASES
1.	English Sole	Hepatocytic Neoplasms	Vancouver Harbor, British Columbia, Canada	0
2.	English Rock Sole Pacific Staghorn Sculpin	Colangiocytic and Hepatocytic Neoplasms	Puget Sound, Washington, USA	10
3.	White Croaker	Epidermal Papillomas Hepatocytic Neoplasms	Southern California Coast, USA	3
4.	Gulf Croaker	Nerve Sheath Neoplasms	Gulf of California, USA	4
5.	Striped Mullet	Fibrosarcomas	Northern Gulf of Mexico (Coastal Texas, Louisiana, Mississippi and Florida) USA	3
6.	Black Bullhead	Epidermal Papillomas	Sewage Treatment Pond Tuskegee, Alabama, USA	3
7.	Brown Bullhead and Yellow Bullhead	Epidermal Papillomas Squamous Carcinomas	Lakes in Polk County Florida, USA	35
8.	Bicolor Damselfish and Grey Snapper	Nerve Sheath Neoplasms	Florida Keys, USA	4
9.	Oyster Toadfish	Hepatocytic Neoplasms	Elizabeth River, Virginia, USA	1

SITE #	FISH	EPIZOOTIC	LOCATION	NUMBER OF RTLA CASES
10.	White Perch	Cholangiocytic and Hepatocytic Neoplasms	Chesapeake Bay, USA	2
11.	White Sucker	Cholangiocytic and Hepatocytic Neoplasms	Deep Creek Lake, Maryland, USA	2
12.	Brown Bullhead	Cholangiocytic Neoplasms	Epsy Bog Pennsylvania, USA	3
13.	Brown Bullhead	Cholangiocytic Neoplasms	Lake Ontoloni, Reading Pennsylvania, USA	0
14.	Brown Bullhead White Perch	Epidermal Papillomas Cholangiocytic Neoplasms	Delaware and Schuykill Rivers, New Jersey and Pennsylvania, USA	11
15.	Brown Bullhead	Cholangiocytic and Hepatocytic Neoplasms	Silver Stream Reservoir, Newburgh, New York, USA	5
16.	Atlantic Tomcod	Hepatocytic Neoplasms	Hudson River, New York, USA	16
17.	Winter Flounder	Hepatocytic Neoplasms	Central Long Island Sound (Dumpsite of of Black Rock Harbor, Bridgeport, Connecticut Dredge Spoils)	0
18.	Winter Flounder	Cholangiocytic and Hepatocytic Neoplasms	Black Rock Harbor, Bridgeport, Connecticut, USA	1
19.	Winter Flounder	Cholangiocytic and Hepatocytic Neoplasms	Narrangansett Bay, Rhode Island, USA	0

SITE #	FISH	EPIZOOTIC	LOCATION	NUMBER OF RTLA CASES
20.	Cunners	Odontogenic Neoplasms	Sakonnet River, Portsmouth, Rhode Island, USA	6
21.	Winter Flounder	Cholangiocytic and Hepatocytic Neoplasms	New Bedford Harbor, Massachusetts, USA	1
22.	Winter Flounder	Cholangiocytic and Hepatocytic Neoplasms	Quincy Bay and Boston Harbor, Massachusetts, USA	9
23.	Brown Bullhead	Pigment Cell Neoplasms	Sudbury River, Massachusetts, USA	10
24.	Brown Bullhead	Hepatocytic Neoplasms	Fox River Watershed, Northern Illinois, USA	0
25.	Sauger and Walleye	Hepatocytic Neoplasms	Torch Lake, Houghton County, Michigan, USA	12
26.	Brown Bullhead	Hepatocytic Neoplasms	Munuscong River, Michigan, USA	5
27.	Bowfin	Hepatocytic Neoplasms	Lower Detroit River, Michigan, USA and Ontario, Canada	1
28.	Brown Bullhead	Cholangiocytic and Hepatocytic Neoplasms Epidermal Papillomas Squamous Carcinomas	Black River, Ohio, USA	154

SITE #	FISH	EPIZOOTIC	LOCATION	NUMBER OF RTLA CASES
29.	Brown Bullhead	Cholangiocytic and Hepatocytic Neoplasms Epidermal Papillomas Squamous Carcinomas	Cuyahoga River, Ohio, USA	24
30.	Brown Bullhead	Epidermal Papillomas Ondontogenic Neoplasms	Inner Harbor of Presque Isle Bay, Lake Erie, Pennsylvania, USA	4
31.	Brown Bullhead	Epidermal Papilloma	Lake Erie Port Rowan, Ontario Canada	0
32.	Slimy Sculpin and White Sucker White Sucker	Cholangiocytic and Hepatocytic Neoplasms Epidermal Papilloma	Western Lake Ontario watershed, Toronto and Burlington, Ontario, Canada	0
33.	Brown Bullhead Freshwater Drum	Cholangiocytic and Hepatocytic Neoplasms Pigment Cell Neoplasms	Lake Erie and tributaries at Niagara, New York, USA	15
34.	Slimy Sculpin	Hepatocytic Neoplasms	Lake Ontario Olcott, New York, USA	1
35.	White Sucker	Cholangiocytic Neoplasms Epidermal Papillomas	Trenton River, Bay of Quinte, Lake Ontario, Ontario, Canada	0
36.	Slimy Sculpin White Sucker	Cholangiocytic Neoplasms Cholangiocytic Neoplasms, Epidermal Papilloma	Ganaraska River, Port Hope, Lake Ontario Ontario, Canada	0
37.	Muskellunge	Lymphosarcoma	Regionally Ubiquitous in Great Lakes	1

SITE #	FISH	EPIZOOTIC	LOCATION	NUMBER OF RTLA CASES
38.	Northern Pike	Lymphosarcoma	Regionally Ubiquitous in Great Lakes	12
39.	Walleye	Dermal Sarcoma	Regionally Ubiquitous in Great Lakes	6
40.	Yellow Perch	Testicular Neoplasms	Regionally Ubiquitous in Great Lakes	0
41.	Common Carp Goldfish Carp/Goldfish Hybrids	Gonadal Neoplasms	Regionally Ubiquitous in Great Lakes	6

Fish

AT = Atlantic tomcod (*Gadus morhua*)
B = Bowfin (*Amia calva*)
BD = Bicolor damselfish (*Pomacentrus partitus*)
BlB = Black bullhead (*Ictalurus melas*)
BrB = Brown bullhead (*Ictalurus nebulosus*)
C = Cunner (*Tautogolabrus adsperus*)
CP = Carp (*Cyprinus carpio*)
ES = English sole (*Parophrys vetulus*
FD = Freshwater drum (*Aplodinotus grunniens*)
G = Goldfish (*Carassius auratus*)
GC = Gulf croaker (*Micropogonias* sp.)
GS = Gray snapper (*Lutjanus griseus*)
M = Muskellunge (*Esox masquinongy*)
NP = Northern pike (*Esox lucius*)
OT = Oyster toadfish (*Opsanus tau*)
PSS = Pacific staghorn sculpin (*Leptocottus armatus*)
RS = Rock sole (*Lepidopsetta bilineata*)
S = Sauger (*Stizostedion canadense*)
SM = Striped mullet (*Mugil cephalus*)
SS = Slimy sculpin (*Cottus cognatus*)
W = Walleye (*Stizostedion vitreum vitreum*)

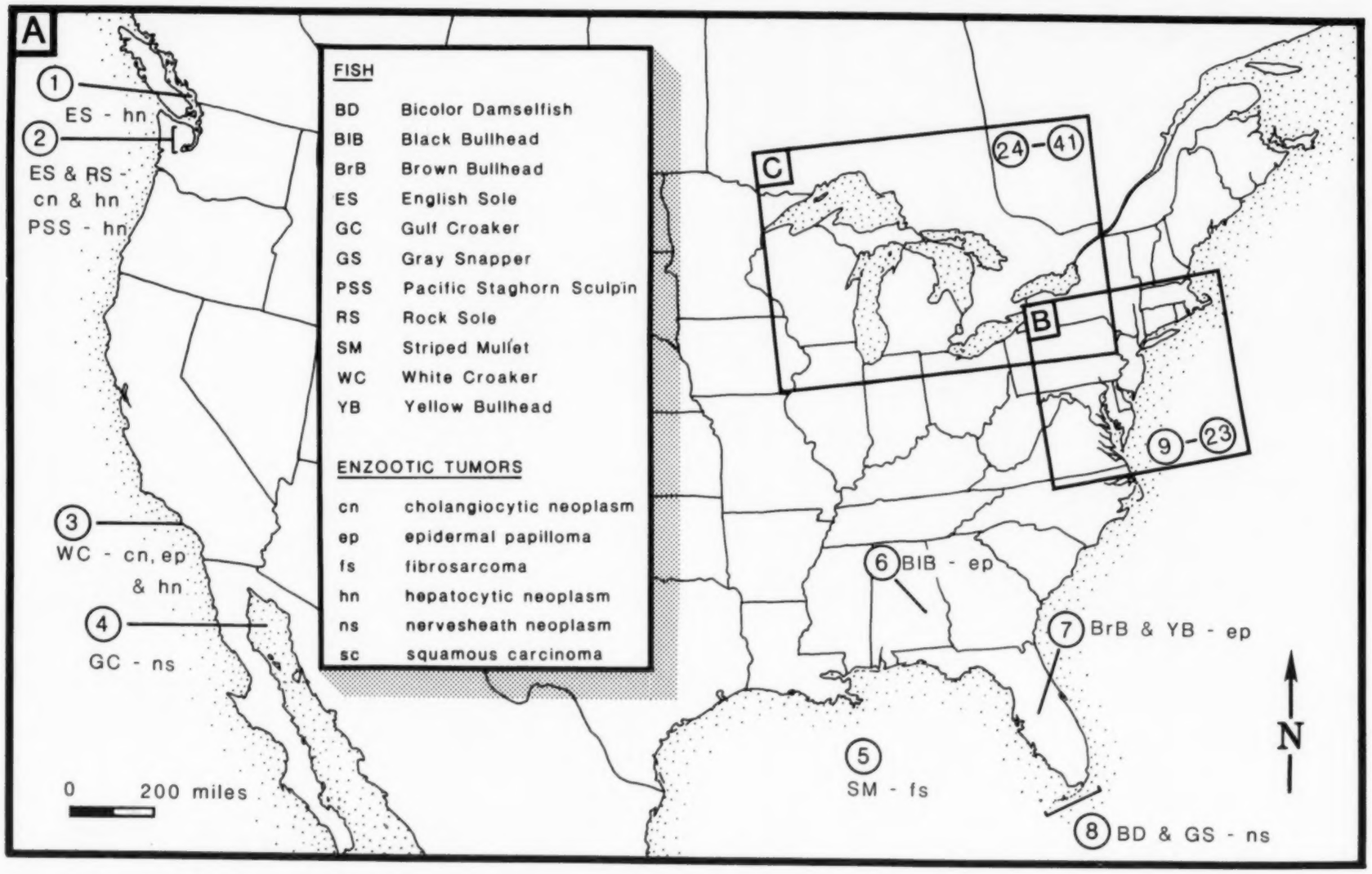

Figure 1A: The location of epizootic neoplasms in wild bony fish numerically designated on a map of North America. The types of neoplasms are designated by lower case letter codes and species of fish are designated by upper case letter codes.

WC = White croaker (*Genyonemus lineatus*)
WF = Winter flounder (*Pseudopleuronectes americanus*)
WP = White perch (*Morone americana*)
WS = White sucker (*Catostomus commersoni*)
YB = Yellow bullhead (*Ictalurus natalis*)
YP = Yellow perch (*Perca flavescens*)

RESULTS

The following entries in the text list the site number (e.g. 1.), the fish species (e.g. ES), the epizootic observed (e.g. hn), and the precise location of the site.

Pacific Northwest

1. ES - hn (Vancouver Harbour, British Columbia)

English sole (*Parophrys vetulus*) with hepatocellular adenoma and hepatocellular foci of alteration from Vancouver Harbour (Goyette et al., 1988).

2. ES & RS - cn & hn, PSS-hn (Puget Sound, northwest Washington)

English sole and rock sole (*Lepidopsetta bilineata*) with cholangioma, cholangiocarcinoma, hepatocellular adenoma, hepatocellular carcinoma and mixed hepatocholangiocarcinoma from the Duwamish Waterway in Elliot Bay (McCain, 1977; McCain et al., 1977) and Eagle Harbor, Everett Harbor, Case Inlet, Port Madison, Denny Way, Mukilteo and the Hylebos Waterway in Commencement Bay (Malins et al., 1987b; Myers et al., 1987).

Pacific staghorn sculpin (*Leptocottus armatus*) with cholangiocellular and hepatocellular neoplasms from waterways in Commencement Bay (Myers and Rhodes, 1988).

Registry Cases (RTLA#s):
416 = Spotted ratfish with myxosarcoma from San Juan Channel.
3085, 3093 = English sole with cholangiocarcinoma and hepatocarcinoma from Duwamish Waterway, Puget Sound.
3086, 3087, 3089, 3090, 3091 and 3092 = English sole with hepatocarcinoma from Duwamish Waterway, Puget Sound.
3293 = English sole with epidermal papilloma from Hylebos Waterway.

Pacific Southwest

3. WC - cn, ep & hn (Southern California coast)

White croaker (*Genyonemus lineatus*) with squamous papilloma of lip from near Los Angeles harbor (Phillips et al., 1976).

White croaker with cholangiocellular carcinoma from Reservation Point, hepatocellular carcinoma from Queensway Bay (both sites in San Pedro Bay), and cholangioma from near the Hyperion outfall (Malins et al., 1987a).

White croaker with squamous papilloma of lip from Santa Monica Bay (Russell and Kotin, 1957; Young, 1964; Harshbarger, 1972).

RTLA#s
62, 404, 405, 495, 549, 1086 = White croaker with papilloma from Orange County, California.
4052 = White croaker with cholangiocarcinoma from Orange County, California.

4. GC - ns (Gulf of California or Sea of Cortez)

Gulf croaker (*Micropogonias* sp.) with melanocytic neurofibroma from between Isla Montaque and Baja California Norte and from one mile offshore of Station Beach, Puerto Penasco, Gulf of California (Barrera et al., 1984).

RTLA#s
1708 = Gulf croaker with melanocytic neurofibroma from between Isla Montaque and Baja California Norte.
1709 = Gulf croaker with melanocytic neurofibroma from Puerto Penasco, Station Beach, approximately one mile offshore.
2297 = Gulf croaker with melanotic neurofibroma from the Gulf of California.
2298 = Gulf croaker with melanotic neurofibroma from the Gulf of California.
2299 = Grunt (*Pomadasys* sp.) with melanotic neurofibroma from near Guaymas, Sonara, Mexico, Gulf of California.

Southeastern U.S. and Northern Gulf of Mexico

5. SM - fs (Northern Gulf of Mexico coast)

Striped mullet (*Mugil cephalus*) with fibrosarcoma and fibroma from Corpus Christi Bay, Texas, Galveston Beach, Texas, Biscayne Bay, Florida, Crystal River, Florida, and Davis Bayou in the Mississippi Sound. (Lightner, 1974; Edwards and Overstreet, 1976; Overstreet, 1988).

RTLA#s
807 and 821 = Striped mullet with ossifying dermal fibroma, Galveston, Texas.
1111 = Striped mullet with ossifying dermal fibroma, Mississippi Sound.

6. BlB - ep (Sewage treatment pond, east central Alabama)

Black bullhead (*Ictalurus melas*) with oral papilloma from the final oxidation pond of the waste water treatment facility in Tuskegee, Alabama (Grizzle et al., 1984; Grizzle, 1985).

RTLA#s
2222 and 2470 = Black bullhead with epidermal papilloma from Tuskegee, Alabama.
2471 = Black bullhead with olfactory sacs carcinoma from Tuskegee, Alabama.

7. BrB - ep & sc; YB ep (Lakes in central Florida)

Yellow bullhead (*Ictalurus natalis*) and brown bullhead (*Ictalurus nebulosus*) with oral epidermal papilloma and squamous carcinoma from Lakes Eva, Agnes, Mattie, Ruth and Mamzor Maniz in Polk County, Florida (Harshbarger, 1972).

RTLA#s
288 = Brown bullhead with squamous carcinoma from Lake Agnes, Polk County, Florida.
289, 384-387, 393, 395, 472, 827, 830, 832-836 and 838 = Brown bullhead with epidermal papilloma from Lake Eva, Polk County, Florida.
328 = Brown bullhead with epidermal papilloma from Lake Mattie, Polk County, Florida.
388 and 389 = Brown bullhead with lipoma from Lake Eva, Polk County, Florida.
392, 394, 475 and 825 = Brown bullhead with squamous carcinoma from Lake Eva, Polk County, Florida.
397-399 = Brown bullhead with epidermal papilloma from Lakes Ruth and Mamzor Maniz, Dundee, Polk County, Florida.
468-471 = Yellow bullhead with epidermal papilloma from Lake Eva, Polk County, Florida.
582 = Yellow bullhead with squamous carcinoma from Lake Eva, Polk County, Florida.
583 = Yellow or brown bullhead with epidermal papilloma from Lake Eva, Polk County, Florida.
584 and 839 = Yellow bullhead with melanoma from Lake Eva, Polk County, Florida.

8. BD & GS - ns (Reef area of Florida Keys)

Bicolor damselfish (*Pomacentrus partitus*) with schwannoma (neurofibromatosis) from the Florida Keys (Schmale et al., 1983; Schmale, 1985; Schmale, 1986).

Gray snapper (*Lutjanus griseus*) with neurilemoma (schwannoma) from the Dry Tortugas and Key West (Lucke, 1942; Schlumberger and Lucke, 1948; and Lucke, 1938). Anecdotal cases include dog snapper (*Lutjanus jocu*), schoolmaster (*Lutjanus apodus*) and slippery dick (*Halichoeres bivittatus*).

RTLA#s
485 = Gray snapper with neurofibroma from Snipe Key, Florida
1378 = Snapper with neurilemoma from Florida
1481 = Gray snapper with invasive neurilemoma from reef area of south Florida.
2289 = Schoolmaster with iridophoroma and neurilemoma from south of Bear Cut, Virginia Key, Florida (Skinner and Kandrashoff, 1988).
3176 = Bicolor damselfish with neurofibrosarcoma (malignant schwannoma) from the Florida Keys.
3177 = Bicolor damselfish with neurilemoma from Triumph Reef, Florida.
3372 = Dog snapper with neurilemoma from Bimini, Bahamas.
3655 = Gray snapper with ossifying dermal sarcoma from a reef off of Miami, Florida.

Mid-Atlantic States and New England

9. OT - hn (Elizabeth River, southeast Virginia)

Oyster toadfish (*Opsanus tau*) with hepatocytic neoplasms from the Elizabeth River, Virginia (Thiyagarajah and Bender, 1988).

RTLA#s
3756 = Oyster toadfish with pancreatic duct adenoma from the Elizabeth River, Virginia.
3901 = Oyster toadfish with hepatocarcinoma and cholangiocarcinoma from the Elizabeth River, Virginia.

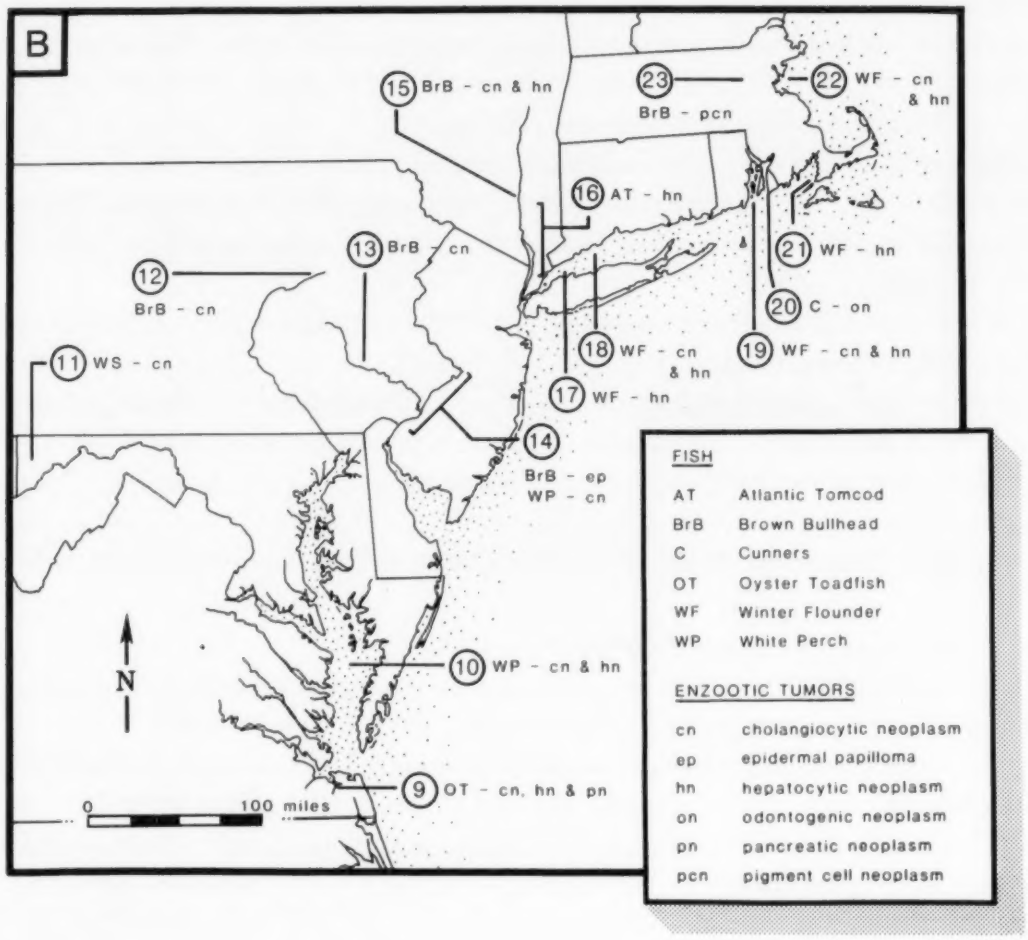

Figure 1B: Inset of map of North America (Figure 1A) showing epizootic neoplasms in the mid-Atlantic coastal area.

3902, 3903 = Oyster toadfish with hepatocellular alteration from the Elizabeth River, Virginia.

10. WP - cn & hn (Chesapeake Bay)

White perch (*Morone americana*) with hepatocarcinoma, cholangioma and hepatocellular alterations from 15 estuarine tributaries of the Chesapeake Bay (May et al., 1987).

White perch with cholangioma from Cedar Point, Maryland (Bunton and Baksi, 1988).

RTLA#s

1225 = White perch with cholangioma from Curtis Creek, Baltimore Harbor, Baltimore, Maryland.

3584 = White perch with cholangiocarcinoma from the Chesapeake Bay.

11. WS - cn & hn (Deep Creek Lake, western Maryland)

White sucker (*Catostomus commersoni*) with cholangiocarcinoma from Deep Creek Lake, Maryland (Dawe et al., 1964; Dawe et al., 1976).

RTLA#s
7 = White sucker with cholangiocarcinoma from Deep Creek Lake, Maryland.
8 = Brown bullhead with hepatocarcinoma from Deep Creek Lake, Maryland.

12. BrB - cn (Epsy Bog, east central Pennsylvania)

Brown bullhead with cholangiocarcinoma from Epsy Bog, Pennsylvania (Putnam and Rice, 1986).

RTLA#s
3180 and 3182 = Brown bullhead with cholangiocarcinoma from Epsy Bog, Pennsylvania.
3181 = Brown bullhead with cholangioma from Epsy Bog, Pennsylvania.

13. BrB - cn (Lake Ontoloni, southeastern Pennsylvania)

Brown bullhead with cholangiocytic neoplasms from Lake Ontoloni, Reading, Pennsylvania. Personal communications with Eric C. May and John J. Black.

14. BrB - ep & hn; WP - cn (Delaware and Schuylkill Rivers, Trenton, New Jersey to Philadelphia, Pennsylvania)

Brown bullhead with epithelioma from streams on the Delaware and Schuylkill Rivers near Philadelphia, Pennsylvania (Lucke and Schlumberger, 1941a; Lucke and Schlumberger, 1941b).

White perch with cholangiocarcinoma and brown bullhead with epidermal papilloma and hepatocellular alteration from the Delaware River at Trenton, New Jersey to below Philadelphia, Pennsylvania. Personal communication with E. Washuta (Anonymous, 1988).

RTLA#s
3883 = White perch with cholangiocarcinoma from Horseshoe Shoals, Delaware River, Pennsylvania (near Navy Yard).
3884 = White perch with cholangiocarcinoma from Penns Landing, Delaware River, Pennsylvania.
3885 = White perch with cholangiocarcinoma from near Petty Island, Delaware River, Pennsylvania.
3886 = White perch with cholangioma from Delaware River, near the mouth of Neshaminy Creek, Pennsylvania.
3880 and 3975 = Brown bullhead with epidermal papilloma and hepatocellular nodule from Paulsboro-Edystone area of the Delaware River, New Jersey.
3881, 3882 and 3976 = Brown bullhead with epidermal papilloma and hepatocellular nodule from the Delaware River at Trenton, New Jersey.
3978 = Brown bullhead with epidermal papilloma and hepatocellular alteration from the Delaware River near the Betsy Ross Bridge, New Jersey.
3980 = Brown bullhead with epidermal papilloma and hepatocarcinoma from the Delaware River, near the Burlington-Bristol Bridge, New Jersey.

15. BrB - cn & hn (Silver Stream Reservoir, Newburgh, New York)

Brown bullhead with hepatocarcinoma and cholangiocarcinoma, schwannoma, cholangioma and squamous carcinoma from Silver Stream Reservoir, Newburgh, New York. Personal communication with Marilyn J. Wolfe.

RTLA#s
3770 = Brown bullhead with hepatocellular alteration, cholangioma, and histiocytic lymphoma from Silver Stream Reservoir, Newburgh, New York.
3772 = Brown bullhead with squamous carcinoma from Silver Stream Reservoir, Newburgh, New York.
3784 = Brown bullhead with schwannoma from Silver Stream Reservoir, Newburgh, New York.
3785 = Brown bullhead with hepatocarcinoma from Silver Stream Reservoir, Newburgh, New York.
3787 = Brown Bullhead with metastatic hepatocarcinoma, cholangiocarcinoma, and schwannoma from Silver Stream Reservoir, Newburgh, New York.
3788 = Brown bullhead with hepatocellular alteration from Silver Stream Reservoir, Newburgh, New York.

16. AT - hn (Lower Hudson River, New York)

Atlantic tomcod (*Microgadus tomcod*) with hepatocellular carcinoma from Verplanck and Garrison on the Hudson River (Cormier, 1986; Cormier, 1989), and from between river miles 25 and 71 of the Hudson River (Smith et al., 1979).

RTLA#s
1868-1871 = Atlantic tomcod with hepatoma and hepatocellular carcinoma from the Hudson River, Buchanan City, New York.
1872 = Atlantic tomcod with hepatocarcinoma from the Hudson River, Buchanan City, New York.
2252-2254, 3107-3109, and 3111 = Atlantic tomcod with hepatocarcinoma from the Hudson River, New York.
2255 = Atlantic tomcod with hepatopancreatocarcinoma from the Hudson River, New York.
2451-2452 = Atlantic tomcod with hepatocarcinoma from the Hudson River, New York.
3110 = Atlantic tomcod with hepatocarcinoma and pancreatic carcinoma from the Hudson River, New York.

17. WF- hn (Central Long Island Sound dump site)

Winter flounder (*Pseudopleuronectes americanus*) with hepatocellular carcinoma from Central Long Island Sound dump site containing dredge spoils from Black Rock Harbor, Bridgeport, Connecticut (Gardner et al. 1987; Gardner et al., 1989; Gardner and Yevich, 1988).

18. WF - cn & hn (Black Rock Harbor, Bridgeport, Connecticut)

Winter flounder with hepatocellular carcinoma from Black Rock Harbor in Bridgeport, Connecticut (Gardner et al. 1987; Gardner et al., 1989; Gardner and Yevich, 1988).

RTLA#
3599 = Winter flounder with hepatocellular alteration from Black Rock Harbor, Bridgeport, Connecticut.

19. WF - cn & hn (Narragansett Bay, southeastern Rhode Island)

Winter flounder with hepatocellular carcinoma from Gaspee Point, in Narragansett Bay, Rhode Island (Gardner and Yevich, 1988; Gardner et al., 1989).

RTLA#

4190 = Winter flounder with seminoma from Fox Island, Narragansett Bay, Rhode Island.

20. C - on (Sakonnet River estuary, southeastern Rhode Island)

Cunner (*Tautogolabrus adspersus*) with odontogenic neoplasms from the Sakonnet River at Portsmouth, Rhode Island (Harshbarger et al., 1976).

RTLA#s

550-552, 675, 923 = Cunner with ameloblastoma from Tiverton, Rhode Island.
553 = Cunner with epidermal papilloma from Tiverton, Rhode Island.

21. WF- cn & pn (New Bedford Harbor, southeastern Massachusetts)

Winter flounder with hepatoma, pancreatic adenoma and a renal neoplasm from New Bedford Harbor, Massachusetts (Gardner and Yevich, 1988; Gardner et al., 1988; Gardner et al., 1989).

RTLA#s

3645 = Winter flounder with cholangiocarcinoma from New Bedford Harbor, Massachusetts.
3990 = Winter flounder with renal papillary cyst adenoma from New Bedford Harbor, Massachusetts.

22. WF - cn & hn (Boston and Quincy Harbors, eastern Massachusetts)

Winter flounder with hepatocarcinoma and cholangiocarcinoma from Deer Island and Quincy Bay in Boston Harbor (Murchelano and Wolke, 1985; Gardner and Pruell, 1988; Gardner et al., 1989).

RTLA#s

3241-3242 = Winter flounder with cholangiocarcinoma from Governor's Island Flats, Boston Harbor, Massachusetts.
3243 = Winter flounder with hepatocellular alteration from Governor's Island Flats, Boston Harbor, Massachusetts.
3424 = Winter flounder with hepatocarcinoma and cholangiocarcinoma from Outer Harbor, Boston, Massachusetts.
3426-3427, and 3434 = Winter flounder with cholangiocarcinoma from Outer Harbor, Boston, Massachusetts.
3428-3429 = Winter flounder with hepatocellular alteration from Outer Harbor, Boston, Massachusetts.

23. BrB - pcn (Concord River watershed, eastern Massachusetts)

Brown bullhead with melanoma (metastatic) from the Sudbury River, Massachusetts. Grossly similar lesions were noted by Thoreau in 1853 and 1858 on brown bullheads from

the Concord and Assabet Rivers, Massachusetts (Torrey, 1906). The Assabet joins the Sudbury to form the Concord.

RTLA#s
4158-4167 = Brown bullhead with metastatic melanoma from Sudbury River, Massachusetts.

24. BrB - hn (Fox River watershed, northern Illinois)
Brown bullhead with hepatocarcinoma from the Fox River Watershed, northern Illinois (Brown et al., 1973).

The Great Lakes and Environs

25. S & W - hn; W - hn (Torch Lake, Upper Peninsula, Michigan)
Sauger (*Stizostedion canadense*) with hepatocarcinoma and walleye (*Stizostedion vitreum vitreum*) with hepatocarcinoma from Torch Lake, Houghton County, Michigan (Black et al., 1982).

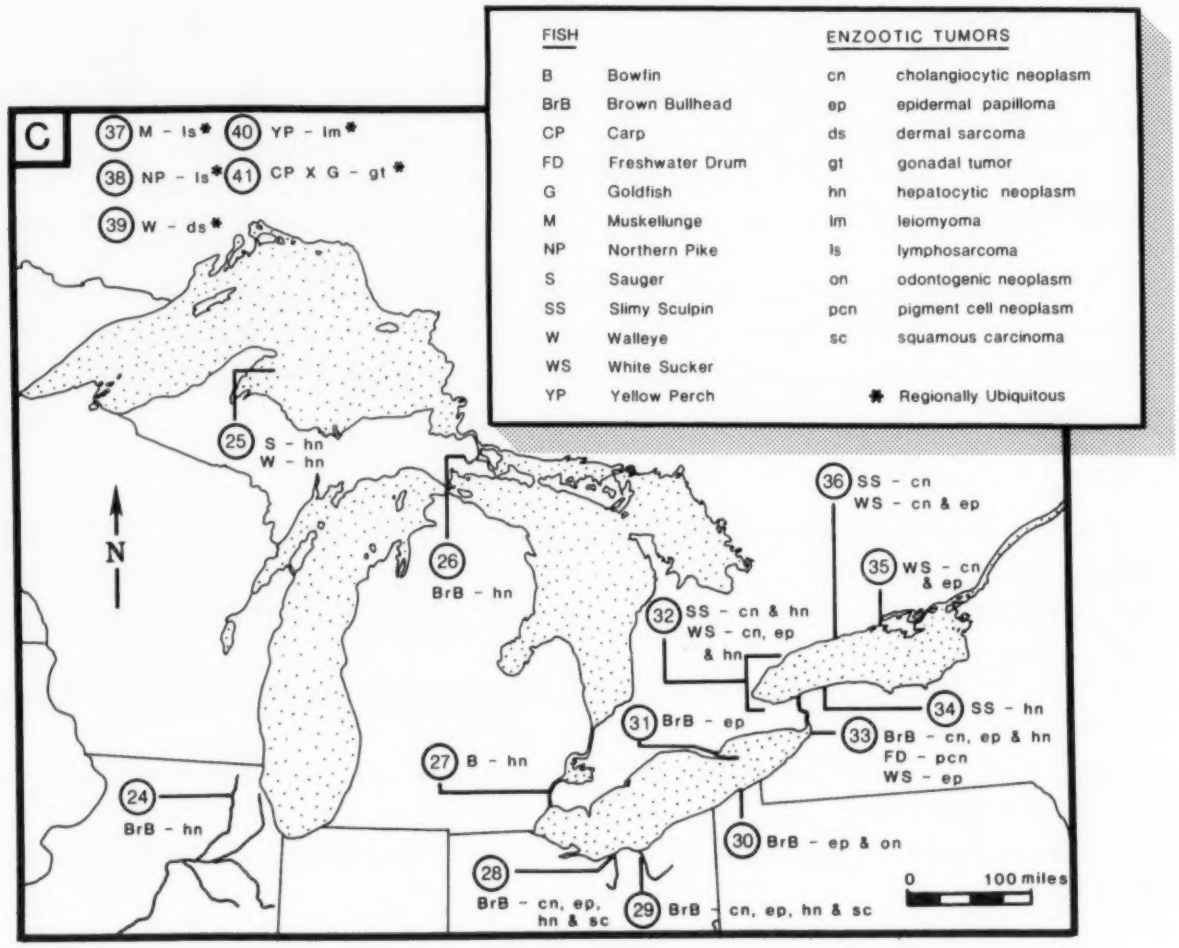

Figure 1C: Inset of map of North America (Figure 1A) showing epizootic neoplasms in the Great Lakes area.

RTLA#s
2257 = Sauger with hepatocarcinoma and leiomyoma from Torch Lake, Houghton County, Michigan.
2407 and 2408 = Walleye with hepatocellular carcinoma from Torch Lake, Houghton County, Michigan.
2409, 2411, 2412, 3046, 3050-3051, 3134, and 3136-3137 = Sauger with hepatocarcinoma from Torch Lake, Houghton County, Michigan.

26. BrB - hn (Munuscong River, Upper Peninsula, Michigan)

Brown bullhead with hepatocellular neoplasms from the Munuscong River, Michigan. Personal communications with Michael J. Mac and Paul C. Baumann.

RTLA#s
3530-3531 = Walleye with hepatocellular alteration from the Munuscong River, Michigan.
3532 = Walleye with hemangioendothelioma from the Munuscong River, Michigan.
3536 and 3538 = Brown bullhead with hepatocellular alteration from the Munuscong River, Michigan.
3539 and 3544 = Brown bullhead with hepatocullar nodule from the Munuscong River, Michigan.
3542 = Brown Bullhead with hepatocarcinoma from the Munuscong River, Michigan.

27. B - hn (Lower Detroit River, Detroit, Michigan, and Windsor, Ontario)

Bowfin (*Amia calva*) with hepatocytic neoplasms, walleye, brown bullhead, white sucker and redhorse sucker (*Moxostoma* sp.) with hepatocytic neoplasms, and freshwater drum (*Aplodinotus grunniens*) with chromatoblastoma from the lower Detroit River. Personal communications with A. E. Maccubbin, and Maccubbin et al. (1987).

RTLA#
3494 = Bowfin with hepatocellular neoplasm from the Trenton Channel of the Detroit River just south of Point Hennepin of Grosse Ile, Michigan.

28. BrB - cn, ep, hn & sc (Black River, Lorain, Ohio)

Brown bullhead with epidermal papilloma, squamous carcinoma, and biliary and hepatic liver lesions, including cholangio- and hepatocarcinoma from the Black River, Lorain, Ohio (Baumann et al., 1982; Baumann and Harshbarger, 1985; Baumann et al., 1987; Baumann et al., this volume).

RTLA#s
2502, 2506, 2508, 2511, 2695, 2700, 2716, 2719, 2723, 3184 and 3255 = Brown bullhead with epidermal papilloma from the Black River, Ohio.
2503 = Brown bullhead with squamous carcinoma and cholangiocarcinoma from the Black River, Ohio.
2504 and 2707 = Brown bullhead with epidermal papilloma, cholangiocarcinoma and hepatocarcinoma from the Black River, Ohio.
2505, 2510, 2623-2624, 2626, 2629, 2696, 2701, 2710, 2715, 2721, 2726, 2729, 3192 and 3253 = Brown bullhead with squamous carcinoma from the Black River, Ohio.

2509, 2512 and 2720 = Brown bullhead with epidermal papilloma and cholangiocarcinoma from the Black River, Ohio.
2513 and 2625 = Brown bullhead with squamous carcinoma, cholangiocarcinoma and hepatocellular alteration from the Black River, Ohio.
2514 = Brown bullhead with epidermal papilloma and cholangioma from the Black River, Ohio.
2622 = Freshwater drum with chromatoblastoma and hepatocarcinoma, from the Black River, Ohio.
2627, 2711, 2725, 2852-2854, 2888-2889, 2922, 2945-2946, 3010 and 3018 = Brown bullhead with cholangiocarcinoma from the Black River, Ohio.
2628 = Channel catfish with hepatocarcinoma from the Black River, Ohio.
2697-2698, 2699, 2705, 2708-2709, 2718, 2722, 2724, 2918-2919, 2921, 2923, 2937, 2947, 2949, 2953, 2973, 2977-2978, 2986, 3013, 3019, 3185 and 3257 = Brown bullhead with cholangiocarcinoma and hepatocarcinoma from the Black River, Ohio.
2702 = Brown bullhead with epidermal papilloma, hepatocarcinoma from the Black River, Ohio.
2703, 2727, 2917, 2925, 2930, 2936, 2939, 2941-2944, 2948, 2950, 2952, 2972, 2974, 2976, 2990-2991, 2996, 3000-3002, 3009, and 3012 = Brown bullhead with hepatocarcinoma from the Black River, Ohio.
2704, 2706, 2714 and 2728 = Brown bullhead with squamous carcinoma and hepatocarcinoma from the Black River, Ohio.
2712 = Freshwater drum with chromatoblastoma from the Black River, Ohio.
2842, 2848, 2951, 2970, 2984, 2988, 2999, 3187 and 3254 = Brown bullhead with cholangiocarcinoma and hepatocellular alteration from the Black River, Ohio.
2849 = Brown bullhead with epidermal papilloma and melanoma from the Black River, Ohio.
2926 = Brown bullhead with hepatocarcinoma and gall bladder adenoma from the Black River, Ohio.
2927, 2932-2934, 2940, 2955, 2989, 2995, 3004, 3014-3017, 3020-3022, 3685, and 3688 = Brown bullhead with hepatocellular alteration from the Black River, Ohio.
2928, 2935 and 2969 = Brown bullhead with cholangioma and hepatocellular alteration from the Black River, Ohio.
2931, 2980, 3005 and 3188 = Brown bullhead with cholangioma from the Black River, Ohio.
2983, 3008, and 3016 = Brown bullhead with cholangioma and hepatocarcinoma from the Black River, Ohio.
2997 = Brown Bullhead with cholangioma, hepatocarcinoma and gall bladder adenoma from the Black River, Ohio.
2998 = Brown bullhead with cholangiocarcinoma, hepatocarcinoma and gall bladder adenoma from Black River, Ohio.
3011, 3026 = Brown Bullhead with cholangiocarcinoma and gall bladder adenoma from the Black River, Ohio.
3025 = Brown bullhead with cholangioma, hepatocellular alteration and gall bladder adenoma from the Black River, Ohio.
3189-3190 = Brown bullhead with squamous carcinoma and hepatocellular alteration from the Black River, Ohio.
3256 and 3259 = Brown bullhead with squamous carcinoma, cholangiocarcinoma and hepatocarcinoma from the Black River, Ohio.

29. BrB - cn, ep, hn & sc (Cuyahoga River, Cleveland, Ohio)

Brown bullhead with squamous carcinoma, hepatocellular neoplasms, cholangiocarcinoma, cholangioma, and epidermal papilloma from the Cuyahoga River, Ohio (Baumann and Mac, 1988).

RTLA#s

3270, 3548, 3549, 3551 and 3569 = Brown bullhead with squamous carcinoma from the Cuyahoga River, Ohio.

3547, 3561, 3563, 3565 and 3700 = Brown bullhead with hepatocellular alteration from the Cuyahoga River, Ohio.

3550 = Brown bullhead with hepatocellular nodule from the Cuyahoga River, Ohio.

3552 = Brown bullhead with hepatocarcinoma from the Cuyahoga River, Ohio.

3553 = Brown bullhead with squamous carcinoma, cholangiocarcinoma and hepatocellular alteration from the Cuyahoga River, Ohio.

3554 and 3558 = Brown bullhead with cholangiocarcinoma and hepatocellular alteration from the Cuyahoga River, Ohio.

3557 = Brown bullhead with cholangioma and hepatocellular alteration from the Cuyahoga River, Ohio.

3559 = Brown bullhead with osteoblastoma from the Cuyahoga River, Ohio.

3567 = Brown bullhead with epidermal papilloma and hepatocellular nodule from the Cuyahoga River, Ohio.

3568 and 3703 = Brown bullhead with epidermal papilloma from the Cuyahoga River, Ohio.

3698-3699 = Brown bullhead with epidermal papilloma and hepatocellular alteration from the Cuyahoga River, Ohio.

3701 = Brown bullhead with hepatocarcinoma and chromatoblastoma from the Cuyahoga River, Ohio.

3705 = Brown bullhead with cholangioma and hepatocellular nodule from the Cuyahoga River, Ohio.

30. BrB - ep & on (Inner harbor of Presque Isle Bay, Lake Erie, Pennsylvania)

Brown bullhead with epidermal papilloma from the Inner Harbor and Upper Bay, and odontoma from the Inner Harbor of Presque Isle Bay, Pennsylvania, Lake Erie (Rice et al., 1986).

RTLA#s

3272 = Brown bullhead with epidermal papilloma and odontoma, Presque Isle Bay, Pennsylvania, Lake Erie.

3273 and 3276-3277 = Brown bullhead with epidermal papilloma from Presque Isle Bay, Pennsylvania, Lake Erie.

31. BrB - ep (Long Point Bay in Lake Erie, Port Rowan, Ontario)

Brown bullhead with epidermal papilloma from Long Point Bay in Lake Erie near Port Rowan, Ontario (Sonstegard, 1978; Smith et al., in press).

32. SS & WS - cn & hn; BrB - ep & cn (Western Lake Ontario and tributaries)

Slimy sculpin (*Cottus cognatus*) with cholangiocytic and/or hepatocytic neoplasms from western Lake Ontario or tributaries, at Burlington, Cobourg and Oakville, Ontario (Fitzsimons, personal communication).

White sucker with cholangiocytic and/or hepatocytic neoplasms from the Humber River, Toronto, Ontario; Sixteen Mile Creek, Oakville, Ontario; the Grindstone Creek and Spencer Creek tributaries to Hamilton Harbour, Ontario (Cairns, V., cited in Black, 1984; Cairns and Ferguson, 1988; Sonstegard, 1977; Smith and Ferguson, 1986; Smith and Zajdlic, 1987; Smith, 1989; Smith et al., in press).

Brown bullhead with epidermal papilloma and cholangiocellular neoplasms in Hamilton Harbour, Ontario (Smith and Ferguson, 1986).

33. BrB - ep, cc & hn; FD - pcn; WS - ep (Eastern Lake Erie and tributaries, Buffalo, New York)

Brown bullhead with epidermal papilloma and freshwater drum with chromatoblastoma from the Buffalo River at the head of the Niagara River, Lake Erie (Black et al., 1980; Cairns, V., cited in Black, 1984).

Freshwater drum with chromatoblastoma, white sucker with oral papilloma from the East Niagara River, mouth of Buffalo River, Smokes Creek, and Frenchman's Creek, Lake Erie (Black, 1983; Cairns, V., cited in Black, 1984).

RTLA#s

1891 = Freshwater drum with neurilemoma from Buffalo Harbor, Buffalo River, New York.

2414 = Freshwater drum with mesothelioma from Lackawanna, Lake Erie, New York.

2415 = Redhorse sucker with gastrointestinal adenocarcinoma from Lackawanna, Lake Erie, New York.

2416 = Freshwater drum with hepatocarcinoma from Tonawanda, Niagara River, New York.

3028 = Brown bullhead with cholangioma, hepatocellular alteration and cystadenoma from the Buffalo River, New York.

3033, 3035-3036 and 3039-3041 = Brown bullhead with epidermal papilloma from the Buffalo River, New York.

3037 = Brown bullhead with cholangiocarcinoma and hepatocarcinoma from the Buffalo River, New York.

3038 = Brown bullhead with epidermal papilloma and hepatocarcinoma from the Buffalo River, New York.

3043 and 3081 = Brown bullhead with hepatocarcinoma from the Buffalo River, New York.

34. SS - hn (Southern Lake Ontario, Olcott, New York)

Slimy sculpin with hepatocytic neoplasms from Lake Ontario, Olcott, New York (Fitzsimons, 1988).

RTLA#

4146 - Slimy sculpin with hepatocellular carcinoma from Lake Ontario, north of Olcott, New York.

35. WS - cn, hn & ep (Bay of Quinte, northeastern Lake Ontario, Trenton, Ontario)

White suckers with cholangiocytic neoplasms and epidermal papilloma from the Trent River at the Bay of Quinte, Trenton, Ontario (Cairns and Fitzsimons, 1988, and personal communication).

36. SS - cn; WS - cn & ep (Tributaries of north central region of Lake Ontario)

Slimy sculpin with cholangiocytic neoplasms from Lake Ontario off Cobourg (Fitzsimons, 1989). White suckers with cholangiocytic neoplasms from Rouge River at Ajax, Ganaraska River at Port Hope, and Cobourg Creek at Cobourg, Ontario (Cairns and Fitzsimons, 1988). White suckers with epidermal papilloma from Colborne, Ontario (Cairns, V., cited in Black, 1984).

37. M - ls (Regionally ubiquitous)

Muskellunge (*Esox masquinongy*) with lymphosarcoma from waterways in Ontario, including the Kawartha Lakes region (Sonstegard, 1975a; Sonstegard, 1975b).

Muskellunge with lymphosarcoma from Lake of the Woods, Ontario (Brown et al., 1973).

RTLA#s

808 = Muskellunge with lymphosarcoma from a waterway in Ontario.

38. NP - ls (Regionally ubiquitous)

Northern pike (*Esox lucius*) with lymphosarcoma from waterways in Alberta, Saskatchewan, Manitoba, Ontario, Quebec, the Northwest Territories, Alaska, Michigan, and New York (Sonstegard, 1975).

Northern pike with lymphosarcoma from the Fox River watershed, northern Illinois and Lake of the Woods area waterways in Ontario (Brown et al., 1973).

RTLA#s

2772 = Northern pike with lymphosarcoma from Douglas Lake, Michigan.
3174 = Northern pike with lymphosarcoma from McGregor Lake, Quebec.
3401, 3402, and 4125-4132 = Northern pike with lymphosarcoma from Washburn County, Wisconsin.

39. W - ds (Regionally ubiquitous)

Walleye with dermal sarcoma from Lakes Oneida, Champlain, Erie and Huron, (Walker, 1969; Bowser et al., 1988).

RTLA#s

3112 and 3113 = Walleye with dermal sarcoma from the Tittabawassee River, Michigan.
3533 and 3535 = Walleye with dermal sarcoma from St. Mary's River, Munuscong Bay, Michigan.
3968 and 3970 = Walleye with dermal sarcoma from Lake Oneida, New York.

40. YP - lm (Regionally ubiquitous)

Yellow perch (*Perca flavescens*) with testicular (rarely ovarian) leiomyoma have been

reported from South Bay, Lake Huron, Black Bay, Lake Superior, Lake Simcoe, Lake Ontario, and several lakes in Algonquin Park, Ontario, including Lake Opeongo and Shrew Lake (Budd and Schroder, 1969; Budd et al., 1975; Budd and Roberts, 1978). Among the thousands of yellow perch sampled in the above locations, single cases of four other tumors were also found: ganglioneuroma, squamous carcinoma, thyroid carcinoma and urinary bladder carcinoma.

41. CP - gt; CP x G - gt; G - gt (Regionally ubiquitous)

Carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), and especially carp x goldfish hybrids with gonadal tumors from the Kindardine area of Lake Huron, from the Rouge River in Detroit, Michigan, and from Toledo, Ohio (Sonstegard, 1977), as well as from Hamilton Harbour, Ontario, and Long Point Bay, Lake Erie (Smith and Ferguson, 1986).

Carp x goldfish hybrids with gonadal tumors from the Welland River, Ontario (Dickman and Steele, 1986).

RTLA#s

934 = Carp with sertoli cell tumor from Great Lakes region of Canada.
1425 = Carp with gonadal leiomyoma versus teratoma from pond in Mystic, Connecticut.
1893 = Goldfish x carp hybrids with gonadoblastoma from near Strawberry Island on the Niagara River, New York.
2843 = Carp with gonadoblastoma from the Black River, Loraine, Ohio.
3660 = Carp x goldfish hybrid with gonadoblastoma from Milwaukee River, Wisconsin.
3781 = Carp with ovarian leiomyomoma from Onandaga Lake, New York.

DISCUSSION

The 41 wild fish tumor epizootics indicated on the map of North America grossly understate the true number by combining multiple proximate sites in such locations as Puget Sound, Lake Ontario, and the Delaware River, and by combining multiple species with epizootic tumors and/or multiple types of epizootic tumors from the same sites.

The epizootics fall into two broad categories: a) hemic, neural, pigment cell, connective tissue, and gonial neoplasms in which occurrence seems unrelated to environmental pollution, and b) epithelial neoplasms (liver, pancreas, gastrointestinal and some epidermal) in which occurrence appears to be strongly correlated with environmental pollution.

Epizootic tumors in the first category include lymphoma in northern pike and muskellunge; nerve sheath cell tumors in goldfish, bicolor damselfish, and various snappers; gonial neoplasms in carp, goldfish, carp/goldfish hybrids, and yellow perch; melanoma in brown bullhead; and dermal fibroma or fibrosarcoma in walleye and striped mullet.

Lymphoma was first reported in northern pike in 1898 by Ohlmacher, prior to pollution of the environment by synthetic organic chemicals. It can have a high prevalence in pristine environments and occurs across North America and in Europe (Mulcahy, 1963; Sonstegard, 1975; Sonstegard, 1976; Ljungberg, 1976; Thompson, 1982). Strong evidence suggesting a viral etiology includes transmissibility by cell free extracts (Mulcahy and O'Leary, 1970; Sonstegard, 1976; Brown et al., 1980), and the presence of reverse transcriptase (Papas et al., 1976; Papas et al., 1977). Similar evidence supports a viral etiology for epizootic lymphoma in the closely related muskellunge (Sonstegard, 1975; Sonstegard, 1976).

Epizootic nerve sheath tumors, that is, neurofibroma and neurilemoma (schwannoma), were also known prior to large-scale industrial production of synthetic organic chemicals, and they occur in areas remote from point sources of pollution, such as the Dry Tortugas and the coral reefs of Florida. However, shipping, military activities, contaminated ground water, and atmospheric fallout are all potential sources of carcinogens in these areas. The diversity and distribution of effected species with a propensity for multicentric familial tumors suggest idiopathic vertical transmission after a germ cell mutation.

The gonadal neoplasms of common carp/goldfish hybrids, which are widely distributed in the Great Lakes area (Sonstegard, 1977; Smith and Ferguson, 1986; Down et al., 1988) have also been reported in Japan (Ishikawa et al., 1976; Ishikawa and Takayama, 1977) and similar tumors have been reported in common carp/Crucian carp hybrids in Spain (Granado-Lorencio et al., 1987). The tumors are encapsulated teratogenic masses which may have a genetic basis. A similar speculation can be made for the widespread gonadal tumors epizootic in yellow perch (Budd and Schroder, 1969; Budd et al., 1975; Budd and Roberts, 1978).

The integumentary fibroma/fibrosarcoma of walleye are widely distributed in pristine, as well as polluted waterways and electron microscopy has revealed virus-like particles (Walker, 1969). Similar lesions (reviewed by Overstreet, 1988) have been found in the striped mullet in various locations along the northern coast of the Gulf of Mexico.

Epizootic neoplasms in the second category, that is, those that appear to be caused or strongly influenced by environmental chemicals, occur in the liver of 13 species and the epidermis of 6 species. The 13 species with epizootic liver tumors are all bottom feeders for at least part of their life history, and in virtually every case, the fish with the tumors are clustered in areas where environmental chemicals are concentrated, as determined by chemical analysis or by proximity to an obvious point source (e.g. effluent outfall, waste dumpsite, military/industrial/municipal development). As further evidence, the deactivation of a coking plant in 1984 has been followed by a decline in the prevalence and severity of liver neoplasms in Black River bullhead in the vicinity of the outfall (P. C. Baumann, M. J. Mac, S. B. Smith, J. C. Harshbarger, unpublished data).

Additional lines of evidence support the conclusion that fish liver neoplasms are caused by carcinogenic chemicals:

a) Historical- The initial discovery of epizootic liver neoplasms in 1964 (Dawe et al., 1964) occurred after the onset (approximately 1940) of the exponential growth of industries producing synthetic organic chemicals. These industries doubled production every 7-8 years. The second liver epizootic in wild fish was reported in 1975 (McCain et al., 1977) and new discoveries have accelerated since.

b) Experimental Carcinogenesis with Pure Compounds - Virtually all of the 2 to 3 dozen known carcinogens tested with one or more fish species have consistently produced liver tumors (Hoover, 1984; Couch and Harshbarger, 1985; Metcalfe, 1989). Numerous other neoplasms have also been induced in experimental carcinogenesis assays with fish, but not as consistently as the liver tumors.

c) Physiology - Fish liver tissue contains the enzymes necessary to metabolize indirect-acting carcinogens to the reactive electrophilic metabolites. These metabolites are capable of producing DNA adducts in fish tissue (Dunn et al., 1987; Varanasi et al., 1989).

d) Experimental Carcinogenesis with Contaminated Sediment- There are several lines of evidence:

1) Extracts of sediment from the Black River, Ohio and the Buffalo River, New York painted or fed to brown bullheads in the laboratory caused tumors and hyperplasias of skin and liver similar to those in the wild (Black, 1983; Black, 1985). Extracts from both locations also caused skin tumors in mice.

2) Winter flounder held four months in tanks with contaminated sediment (from Central Long Island Sound dumpsite) and fed contaminated blue mussels (*Mytilus edulis*) exhibited nesidioblastosis, intestinal regenerative hyperplasia and polyps, and hepatocytic cytomegaly (Gardner et al., 1987; Gardner et al., 1988). These lesions were similar to those observed in winter flounder caught near the dumpsite, itself.

3) Extracts of sediment from Hamilton Harbour in western Lake Ontario injected into rainbow trout sac fry induced liver tumors (Metcalfe et al., 1988).

e) Spontaneous Liver Neoplasms- Spontaneous liver tumors are exceedingly rare in wild fish from clean habitats and control fish in carcinogenicity experiments.

The six species with epizootic epidermal neoplasms also occur in chemically polluted environments. Epizootic odontogenic neoplasms in cunners have only been reported from the industrialized Sakonnet River, Rhode Island (Harshbarger et al., 1976). White croaker with oral papillomas have been found in the vicinity of the Santa Monica and the Santa Anna sewage outfalls, but were not found on white croaker surveyed in the unpolluted waters of Catalina Island (Russell and Kotin, 1957). Similarly, black bullheads living in, or transplanted to the final oxidation pond of a sewage treatment facility exhibited oral papilloma. Once the level of chlorination was reduced, new lesions did not occur (Grizzle et al., 1984; Grizzle, 1985). The prevalence of papilloma in white sucker varies throughout the Great Lakes, and other waterways from Alberta to Quebec. In Lake Ontario and environs, the prevalence ranges from zero in one unpolluted site (Cairns, V., personal communication) to approximately 60% in heavily polluted areas (Smith et al., in press). Virus-like particles were once reported in white sucker papilloma (Sonstegard, 1973), but this appears to have been an error because the same electron photomicrograph was republished (Sonstegard, 1976) as an example of virus-like particles in a northern pike epidermal hyperplastic plaque. These particles resemble the virus-like particles reported by Winqvist (1968) in plaque-like epidermal hyperplasia of northern pike. Subsequent electron microscopy has not revealed virus particles in the white sucker papilloma (Smith et al., 1989). On the other hand, epidermal papillomas have been shown to develop in white suckers kept in presumably unpolluted well-water (Smith and Zajdlik, 1987), so, while chemical pollutants appear to be an "influencing factor", a viral etiology cannot be ruled out.

Viruses have also been reported to be present in oral epidermal papilloma of the European eel (Christiansen and Jensen, 1950), and epidermal papilloma in the European smelt (Anders and Moller, 1985), but have not been shown to be the causative agent. However, Sano et al. (1985) presented experimental data implicating a herpesvirus as the causative agent of carp pox (papilloma), and Sano et al. (1983) presented data implicating a herpes-like virus as the causative agent for a papilloma (RTLA# 3394) in Yamame salmon (*Oncorhynchus masou*).

Epidermal papillomas were reported almost 50 years ago in 166 brown bullhead specimens from the Delaware and Schuylkill Rivers at Philadelphia (Lucke and Schlumberger, 1941a; Lucke and Schlumberger,1941b), and they still occur there (Anonymous, 1988). Papillomas and squamous carcinomas were also reported in the 1970s

in bullheads from Lake Eva and several other lakes in central Florida in the heart of the citrus growing area (Harshbarger, 1972). More than 50 pesticides, many of which were organochlorines, were in use in the citrus groves at that time, plus the lakes were sprayed with 2,4-D, paraquat, and diquat to kill water hyacinths. Papillomas and squamous carcinomas have been found in the Black River, Ohio, and in a number of the other locations where liver neoplasms have been found. However, a statistical analysis by Baumann et al. (1987) showed that the epidermal lesions in Black River bullheads occurred independently of the liver neoplasms. Most convincing of a chemical etiology, sediment extract from the Black and the Buffalo Rivers painted on brown bullheads in the laboratory caused epidermal proliferation after nine months of treatment (Black River extract) and epidermal papillomas after 12 months of treatment (Buffalo River extract). The extracts also induced skin papillomas on mice. Therefore, the exact etiology of epidermal papillomas in fish species remains unclear.

In conclusion, epithelial tumors in fish, and specifically hepatocellular neoplasms, are strongly correlated with exposure to chemical contaminants. Carcinogenic risk appears to be related to life history, with bottom dwelling species being the most commonly affected. However, the propensity of some species to tumor development (e.g. brown bullheads, English sole) suggests that certain fish species may be more sensitive to chemical carcinogenesis than others.

ACKNOWLEDGEMENTS

The authors would like to thank Vic Cairns, John Fitzsimons, and Ian Smith for suggestions and comments on the manuscript. Thanks to Miss Phyllis Spero for her assistance in preparing the manuscript and verifying references.

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The Science of the Total Environment, 94 (1990) 33–50
Elsevier

OVERVIEW OF STUDIES ON LIVER CARCINOGENESIS IN ENGLISH SOLE FROM PUGET SOUND; EVIDENCE FOR A XENOBIOTIC CHEMICAL ETIOLOGY I: PATHOLOGY AND EPIZOOTIOLOGY

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ABSTRACT

Livers of wild English sole (Parophrys vetulus) from polluted waterways and embayments of Puget Sound, Washington, are affected by a spectrum of multiple, co-occurring idiopathic hepatic lesions, including neoplasms, putative preneoplastic foci of cellular alteration, and unique degeneration conditions. Results from a statistical analysis of the patterns of co-occurrence of these lesions in wild English sole indicate that these lesions represent morphologically identifiable steps leading to the development of hepatic neoplasms. This sequence parallels the lesion progression in experimental models of chemically induced liver carcinogenesis in rodents. The hypothesis that these lesions in wild English sole can be caused by exposure to certain xenobiotic hepatotoxic and hepatocarcinogenic compounds in Puget Sound is based on: a) statistical associations between levels of aromatic hydrocarbons (Σ AHs) in sediment and prevalences of these idiopathic liver lesions, b) the contribution of Σ AHs in accounting for the variability in hepatic neoplasm prevalence in a logistic regression model, c) elevated odds ratios for several idiopathic hepatic lesion types in sole from polluted sites in Puget Sound, d) significant correlations between prevalences of idiopathic hepatic lesions and levels of fluorescent metabolites of aromatic compounds (FACs) in bile of English sole, and e) experimental induction of putatively preneoplastic focal lesions in English sole injected with a PAH-enriched fraction of an extract from a contaminated urban sediment from Puget Sound, that were morphologically identical to lesions found in wild English sole from the same site.

INTRODUCTION

Since the mid-1970s, we have been conducting research on the pathology of fish in Puget Sound, Washington, and elsewhere along the western coast of the USA, using a multidisciplinary approach to the study of deleterious effects in wild fish resulting from exposure to xenobiotic chemicals in the environment. This approach combines histopathologic examination of resident fish with chemical analyses of sediment and various fish tissues, including bile. We utilize bottom-dwelling marine fish species, which are in nearly continuous contact with the bottom sediments and feed on bottom organisms, as

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sentinel organisms for exposure to the xenobiotic chemicals often present in these sediments.

The impetus for this approach came in 1975 when significant prevalences of hepatic neoplasms were first found in English sole (*Parophrys vetulus*) from the Duwamish Waterway, an urban waterway in Seattle, Washington that receives industrial, municipal, and agricultural wastes (McCain et al., 1977; Pierce et al., 1978). The bottom sediments in this waterway contain high levels of carcinogenic polycyclic aromatic hydrocarbons (PAHs), including benzo(a)pyrene and benzo(a)anthracene, as well as heavy metals, polychlorinated biphenyls (PCBs), and other chlorinated hydrocarbons (Malins et al., 1984). Studies subsequent to the original reports of neoplasms in English sole from the Duwamish Waterway have further assessed the hepatic histopathology of several demersal marine fish species from over 45 sampling stations in Puget Sound; sites that show a broad range of sediment pollutant profiles (Malins et al., 1980; Malins et al., 1982; Malins et al., 1984; Malins et al., 1985a; Malins et al., 1985b; Malins et al., 1987; Malins et al., 1988; McCain et al., 1982; Myers et al., 1987).

Because the liver is well known as a target organ for the effects of xenobiotic chemical toxicants and carcinogens in both mammals (Plaa, 1986) and fish (Meyers and Hendricks, 1982), we have focused our efforts on detection of toxicopathic liver lesions in wild fish species. The lesions observed resemble those induced in mammals (Frith and Ward, 1980; Jones and Butler, 1975; Jones and Butler, 1978), or fish (Hendricks et al., 1984) by controlled exposure to toxicants and carcinogens, and thereby represent potential indicators of such exposure. The main advantage of this approach, when combined with suppporting data of exposure and uptake of xenobiotic chemicals, is that the presence in wild fish of liver lesions that are morphologically identical to those induced by hepatotoxicants and/or hepatocarcinogens in laboratory exposure studies provides a fairly direct method of initially assessing the potential sublethal effects of xenobiotic compounds on wild fish. This paper represents a brief summary of our results, obtained from both field and laboratory studies, that indicate a xenobiotic chemical etiology for certain hepatic lesions detected in English sole from Puget Sound, Washington.

METHODS OF FIELD RESEARCH

The general approach we have employed is to first select sampling stations from urban areas proximate to multiple pollutant sources such as sewer outfalls, storm drains, or industrial effluents. These areas must also contain sediments with a depositional character; that is, with a high mud-to-sand ratio, since these sediments are the most likely to show high levels of xenobiotic chemicals due to their adsorption to fine particulate matter. Also selected are stations with depositional sediments that are remote from obvious pollutant sources, thus serving as reference sites. Target fish species must have a broad geographical distribution within the study area, be bottom-dwelling, feed on botton-dwelling organisms, and show minimal migration outside the site of capture. Through experience, we have also learned to concentrate on adult specimens of the target species, generally more than one year in age, because these older fish show higher prevalances of toxicopathic liver lesions than juveniles (Rhodes et al., 1987).

The sampling protocol for this type of study involves capture of fish by otter trawl, with collection of generally at least 30 specimens of each species per site and documentation of

basic biological information, including fish length, gender, and age. As part of the necropsy protocol, bile is collected for determination of levels of metabolites of various PAHs (Krahn et al., 1984; Krahn et al., 1986; Krahn et al., 1987). It is imperative to analyze for the metabolites of these compounds because the parent PAHs are rapidly metabolized in the fish after uptake (Varanasi et al., 1989). We also collect and preserve excised portions of the major organs for histopathological examination. However, experience has shown that the liver is the organ most often affected with potentially toxicopathic lesions. We also collect surface sediment samples and samples of fish tissue, such as liver or muscle, for analysis of the levels and types of xenobiotics. The histopathological lesions, however, are the primary indicators of biologic effects of contaminant exposure for most of our field studies, and certain lesion types have proven to be reliable biological indicators of the toxic and carcinogenic effects of such exposure.

OVERVIEW OF HEPATIC LESIONS IN WILD ENGLISH SOLE

In terms of the suspected toxicopathic liver lesions detected, the English sole has turned out to be our most effective indicator species, and displays a spectrum of multiple, co-occurring idiopathic liver lesions (Table 1) that closely parallels the types of hepatic lesions induced experimentally by chemical hepatotoxicants and hepatocarcinogens in the mouse, rat, and certain fish species (Myers et al., 1987). These conditions are found at significantly higher prevalences in English sole captured from highly polluted urban estuaries and embayments in Puget Sound than in sole from relatively unpolluted reference areas (Malins et al., 1984; Malins et al., 1985a; Malins et al., 1985b; Malins et al., 1987; Malins et al., 1988; McCain et al., 1982; Rhodes et al., 1987). This geographic pattern of lesion distribution is similar to that found for other bottom-dwelling species in Puget Sound, such as rock sole, *Lepidopsetta bilineata* and starry flounder, *Platichthys stellatus* (Myers and Rhodes, 1988). The prevalences of significant idiopathic liver lesions, especially neoplasms are, however, much higher in the English sole.

The pathologic anatomy of these lesion types has recently been described in detail (Myers et al., 1987). The most frequently encountered idiopathic lesion in the liver of English sole, appearing first in young of the year or juvenile fish (Rhodes et al., 1987) is a unique, nonproliferative degenerative condition characterized by a marked increase in nuclear and cellular diameters in affected hepatocytes, nuclear hyperchromasia, and various cytoplasmic degenerative changes, including hyalinization. Since there is no inflammatory response, this lesion is referred to as megalocytic hepatosis. This lesion and the related precursor condition, nuclear pleomorphism, are identical to the early changes induced by a spectrum of hepatotoxicants and hepatocarcinogens in rodent models (Myers et al., 1987), and are interpreted as manifestations of the chronic to subchronic hepatotoxicity of these compounds. This cytotoxicity is known to be an essential early step in rodent hepatocarcinogenesis, and functions to provide a stimulus for the compensatory proliferative response that actually "fixes" the molecular lesions in the hepatocellular DNA responsible for neoplastic initiation and transformation (Tsuda and Farber, 1980).

The lesion occurring most frequently with megalocytic hepatosis is hepatocellular regeneration (Myers et al., 1987), representing a compensatory proliferative response to the degeneration and necrosis seen in megalocytic hepatosis. This compensatory proliferative response is also a necessary step in the process of hepatic neoplasia (Ying et al., 1981;

Table 1. Important Idiopathic Hepatic Lesions in English Sole

Lesion type/category	
1) hepatocellular necrosis/degeneration	(nonspecific, degenerative--includes spongiosis hepatis)
2) nuclear pleomorphism	(unique,degenerative)
3) megalocytic hepatosis	(unique,degenerative)
4) hepatocellular regeneration	(proliferative,non-neoplastic)
5) clear cell focus of cellular alteration	(preneoplastic)
6) eosinophilic focus " "	(preneoplastic)
7) basophilic focus " "	(preneoplastic)
8) liver cell adenoma	(benign neoplasm)
9) hepatocellular carcinoma	(malignant neoplasm)
10) cholangioma	(benign neoplasm)
11) cholangiocellular carcinoma	(malignant neoplasm)
12) mixed hepatobiliary carcinoma	(malignant neoplasm)
13) cholangiofibrosis	(proliferative, non-neoplastic)
14) hemosiderosis	(storage disorder)
15) steatosis/fatty change	(storage disorders)

Columbano et al., 1981). These regenerative cells may be the population of proliferative cells from which initiated hepatocytes develop, according to the two stage initiation-promotion model of hepatocarcinogenesis, and may represent a population of cells that are resistant to the cytotoxic effects (Solt et al., 1977) of the carcinogens present in the polluted sediments in which English sole reside.

Also frequently detected with megalocytic hepatosis and regenerative islands are discrete, spherical foci or micronodules of three basic types (Myers et al., 1987). These foci of cellular alteration generally first appear in sole at least one year in age (Rhodes et al., 1987), and are considered to be preneoplastic lesions in rodent hepatocarcinogenesis models. These foci, from which true neoplasms may develop under the correct conditions, represent an obligatory precursor step in the induction of hepatic neoplasms (Farber and Cameron, 1980). The three basic types of foci seen in English sole are the eosinophilic,

basophilic, and clear cell focus, based on their tinctorial qualities in the standard hemotoxylin and eosin stain. All three types can be recognized histochemically by a reduction or absence of cytoplasmic iron (Myers et al., 1987), a histochemical feature shared with similar focal lesions in rodent hepatocarcinogenesis models (Williams, 1982). These focal lesions in English sole rarely contain other hepatic elements such as bile ducts, pancreatic acini, blood vessels, or macrophage aggregates. The relationship among these foci types, in terms of their role and fate in hepatocarcinogenesis, is not yet well understood. However, the basophilic focus is the focal lesion believed most proximate, in the temporal sense, to the development of true hepatocellular neoplasms (Bannasch et al., 1980), and is considered a microcarcinoma in the rainbow trout hepatocarcinogenesis model (Hendricks et al., 1984). Hepatocytes composing similar focal lesions in rodents are also considered to be resistant to the cytotoxic effects of hepatocarcinogens (Solt et al., 1977).

High prevalences (up to 25%) of hepatic neoplasms are encountered in adult English sole of generally at least two years in age (Rhodes et al., 1987). These lesions are found almost strictly in fish from polluted sites within Puget Sound, and are usually quite obvious grossly, with multiple nodules often forming the bulk of the liver. The basic histologic types of hepatic neoplasms are classified strictly on their morphologic appearance, since we have little information on their biological behavior and no information as to the clinical outcome for affected fish. Liver cell adenomas are characterized by compression of the surrounding parenchyma, well defined separation of proliferative tissue from normal tissue, fairly normal architecture, and relative absence of other hepatic elements. Liver cell adenomas are usually basophilic, but eosinophilic and vacuolated variations do occur. As is the case for all hepatocellular tumors in English sole, the neoplastic hepatocytes are deficient in iron accumulation (Myers et al., 1987).

Hepatocellular neoplasms with widely accepted histologic features of malignancy are also found in English sole. These hepatocellular carcinomas are typically multiple, with irregular borders, and show compression and invasion. They usually have an irregular trabecular architecture and are composed of polygonal, pleomorphic, anaplastic, and occasionally vacuolated hepatocytes showing loss of cellular polarity with respect to their orientation toward the sinusoids, and often containing a uniquely fibrillar cytoplasm (Stehr et al., 1988). Areas of hepatocytes with a cytomorphology typical of carcinomatous cells are occasionally found within adenomas. These "nodules within nodules" suggest that transition from an adenoma to a carcinoma is one possible mode of neoplastic progression in English sole (Myers et al., 1987).

A separate class of hepatic neoplasm is clearly biliary in origin and is characterized by a proliferation of biliary epithelium assuming relative degrees of tubular organization (Myers et al., 1987). Well organized tumors of this type (cholangiomas) are rare, and typically we detect cholangiocellular carcinomas featuring indistinct, disorganized tubular profiles and showing irregular borders with invasion of the surrounding parenchyma. Malignant neoplasms of this type often replace high proportions of the hepatic parenchyma, and can metastasize (Myers et al., 1987). Occasionally found are carcinomas with a mixed biliary and hepatocellular origin, whereas mesenchymal neoplasms in the English sole liver are rare.

No evidence for a viral etiology for any of these lesions has been shown through ultrastructural observations with transmission electron microscopy (Stehr et al., 1988; Stehr, unpublished observations).

PATTERNS OF HEPATIC LESION CO-OCCURRENCE

The spectrum of multiple, co-occurring idiopathic liver lesions found in English sole (Myers et al., 1987) closely parallels the degenerative, regenerative, preneoplastic, and neoplastic hepatic lesions induced experimentally by chemical hepatotoxicants and hepatocarcinogens in the mouse and rat (Farber and Sarma, 1987), and in certain fish species (Hendricks et al., 1984; Couch and Courtney, 1987). To clarify the role of these lesion types within the context of liver neoplasia in English sole, we conducted a three-way chi-square contingency analysis of lesion type co-occurrence in English sole from Eagle Harbor, Washington (Myers et al., 1987). This small embayment in Puget Sound has sediments highly polluted by PAHs (Malins et al., 1985b) that originate from a nearby creosote impregnation and piling treatment plant (Krone et al., 1986). The inferences from this analysis are that lesions co-occurring at levels of statistical significance may be caused by associated etiologic agents, or be temporally related to one another, or both. A possible temporal association includes involvement in a series of lesions progressing towards hepatic neoplasms, as has been clearly demonstrated in various rodent hepatocarcinogenesis models (Farber and Sarma, 1987).

The schematic diagram in Figure 1 presents the results of this analysis, which represents an attempt to make some temporal sense of the profusion of nonsynchronized, multiple

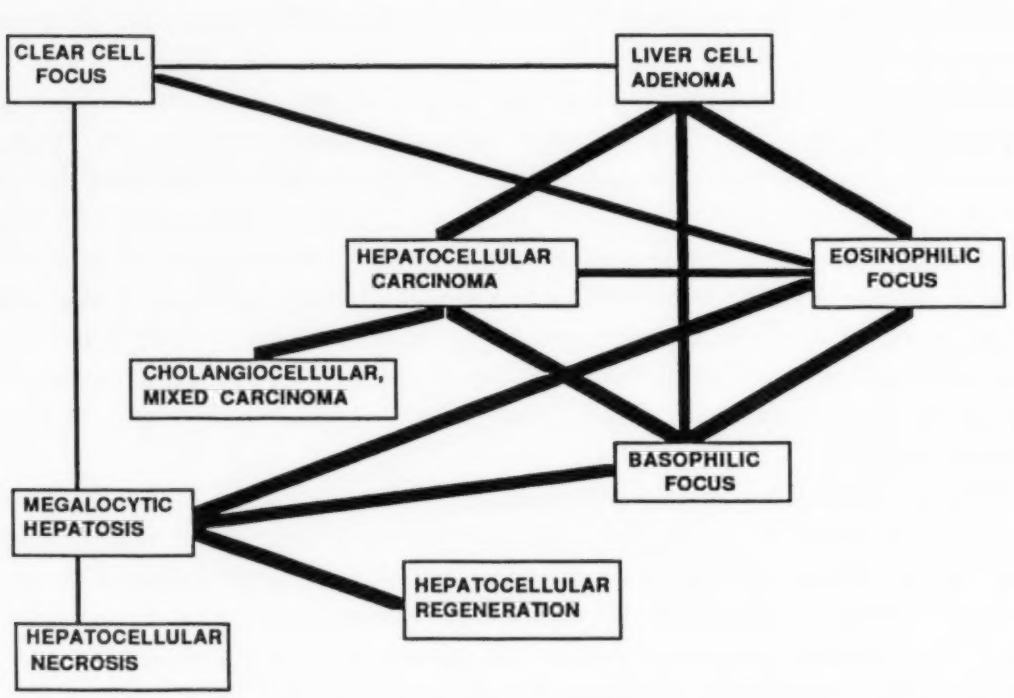

Figure 1: Histogenic relationships (associations determined by 3-way contingency analysis) among idiopathic lesions involved in hepatic neoplasia in feral English sole. Lines between lesion types indicate significant associations between those lesions, in terms of their frequency of co-occurrence. Thickness of lines shows the relative consistency of that association. Diagram adapted from Myers et al. (1987).

hepatic lesion types we typically encounter, and which is consistent with what one would expect under a situation of continuous exposure to chemical hepatocarcinogens in the environment. The lines between lesion types indicate a statistically significant degree of association, with the thicker bars referring to the more statistically consistent associations, and, hence, lesions that are more closely related in terms of their temporal histogenesis. The pivotal position of megalocytic hepatosis indicates that this condition is the initial hepatotoxic, degenerative lesion in the histogenesis of liver neoplasia in the English sole. It is associated with other degenerative/necrotic conditions, with hepatocellular regeneration, and also with eosinophilic, basophilic, and clear cell foci. We believe that the regenerative and focal lesions follow this initial hepatotoxic lesion, with regeneration being a compensatory proliferative response to the necrosis and degeneration seen in megalocytic hepatosis.

There are strong and consistent associations among all of the putatively preneoplastic foci of cellular alteration (basophilic, eosinophilic, and clear cell foci), between these focal lesions and the different types of neoplasms, and among the various neoplasm types. The very consistent association between the basophilic focus and both types of hepatocellular neoplasms suggests that the basophilic focus is temporally proximate to the development of hepatocellular neoplasms in English sole. This interpretation is in accord with the role of the basophilic focus in rodent models (Bannasch et al., 1980) and in the rainbow trout model (Hendricks et al., 1984) of hepatocarcinogenesis. The lack of an association between megalocytic hepatosis and neoplasms indicates that these lesions are sufficiently separated in the temporal sequence of hepatic neoplasia that they tend not to co-occur together. To summarize, this analysis, in conjunction with data on age of occurrence for the various lesions types (Rhodes et al., 1987), strongly suggests that the lesions involved in the multistep process of hepatic neoplasia in English sole do comprise a progressive sequence that parallels the histogenesis of liver neoplasia in mouse, rat, and, to a lesser extent, rainbow trout. These results are the first confirmation of the experimentally derived histogenesis of chemically induced hepatic neoplasia in any vertebrate population exposed to hepatotoxicants and hepatocarcinogens in the environment. Considering the associational patterns among the lesion types shown in this model, the importance of enumerating hepatic lesion types other than frank neoplasms as potential indicators of contaminant exposure is also underscored (Myers et al., 1988).

RELATIONSHIPS BETWEEN HEPATIC LESIONS AND CONTAMINANT EXPOSURE

Aside from the results presented thus far supporting the hypothesis that these lesions are the result of exposure to environmental contaminants by virtue of the morphologic similarity to lesions induced by experimental exposure to hepatotoxicants and carcinogens, we have documented several strong statistical associations between these lesions and exposure to chemical contaminants in the environment. First, there is the unique distribution of these conditions, especially the neoplasms, among the sampling sites in Puget Sound. With few exceptions, these idiopathic liver conditions are found only in estuaries and embayments in proximity to urban centers or major pollutant sources (Malins et al., 1984; Malins et al., 1985a; Malins et al., 1985b; Malins et al., 1987; Malins et al., 1988; McCain et al., 1982). More direct associations of these lesions with contaminant exposure have been demonstrated by statistical comparison of idiopathic lesion prevalences with actual

concentrations of sediment-associated aromatic hydrocarbons (Σ AHs) using the Spearman rank correlation method (Malins et al., 1984). Significant correlations have been shown between prevalences of: a) all idiopathic liver lesions and summed concentrations of Σ AHs (Malins et al., 1984), b) hepatic neoplasms and Σ AHs (Malins et al., 1984, Malins et al., 1988), c) foci of cellular alteration and Σ AHs (Table 2), and d) megalocytic hepatosis and Σ AHs (Table 2).

While the strength (p value) of a particular association or correlation is important in establishing the significance of the relationship between variables, of greater importance is

Table 2. Correlations between aromatic hydrocarbon concentrations in sediment and the occurrence of hepatic neoplasms (**NEO**), putative preneoplastic lesions (**PRE**) and megalocytic hepatosis (**MH**), respectively. The adjusted significance level takes into account the number of individual statistical tests performed.

Study	Period	#Stations	# Fish Examined	r_sNEO[a] (Sig)	r_sPRE (Sig)	r_sMH (Sig)
1	'79-80	31	1823	0.48 (0.003)	0.47 (0.004)	0.54 (0.001)
2	'82	4	200	0.60 (0.200)	0.00 (0.500)	0.80 (0.100)
3	'83	2	66	1.00 (0.500)	1.00 (0.500)	1.00 (0.500)
4	'83-84	11	229	0.35 (0.148)	0.00 (0.500)	0.36 (0.136)
5	'84	4	74	1.00 (0.001)	0.80 (0.100)	0.80 (0.100)
6	'84	9	265	0.54 (0.065)	0.54 (0.065)	0.23 (0.273)

	NEOPLASMS	PRENEO	MH
number of studies in agreement	6	6	6
Fisher's combined probability stat.	39.296	25.274	30.999
degrees of freedom	12	12	12
adjusted significance level	0.013	0.013	0.013
significance[b]	**0.0001***	**0.014**	**0.002***

[a] Correlation coefficient from the Spearman rank correlation method

* Indicates significance was below the critical value (adjusted significance level).

[b] From Fisher's Combined Probability Test

the consistency of that relationship among several studies of a similar design. The same relationships discussed above have been consistently identified in multiple studies within Puget Sound, as shown by the Fisher's combined probability test, which tests the consistency among multiple field studies of the statistical association between the prevalence of these lesion types and Σ AH concentrations in the sediment (Table 2).

A logistic regression model has been recently constructed (Varanasi et al., 1987b; Schiewe et al., 1988) that utilizes data from multiple studies in Puget Sound. The model incorporates data on neoplasm prevalence, sediment contaminant levels, interactions among different classes of sediment contaminants, and fish size (an indirect measure of fish age) in an attempt to apply analytical epidemiology to the study of liver neoplasia in English sole and its relation to contaminant exposure (Figure 2). The results from this approach further support the hypothesis that PAHs are a major etiologic agent of liver neoplasms in English sole. The variation in levels of PAHs in sediment accounts for approximately 12% of the variation in prevalence of hepatic neoplasms; far more than for any other identified contaminant group. The model also indicates that there is a significant positive effect of fish size (age) on neoplasm prevalence, with a slight positive effect contributed by

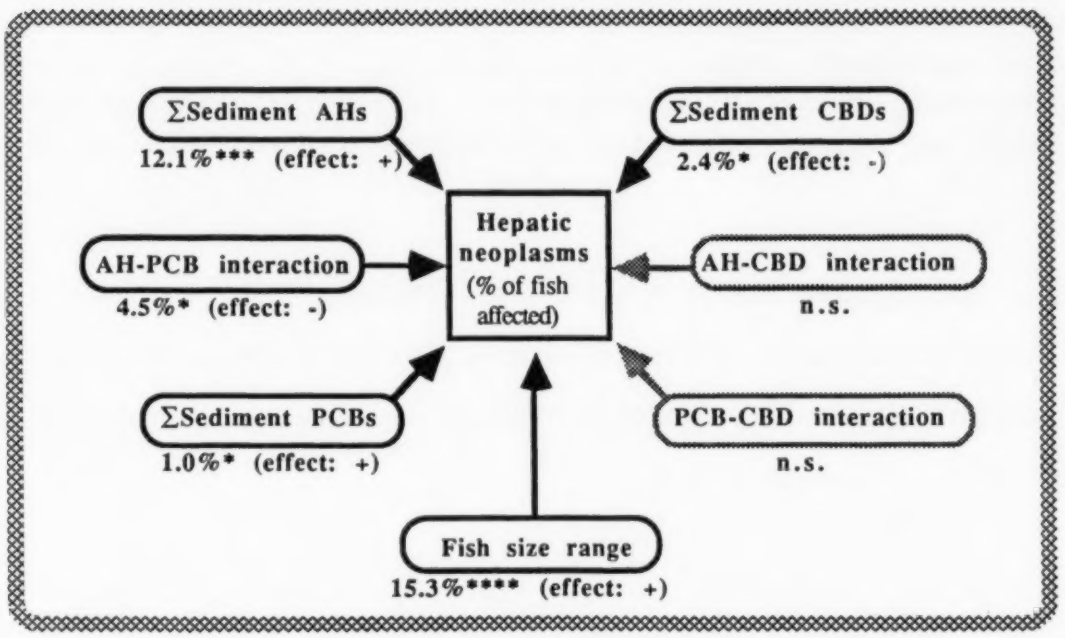

Figure 2: A model summarizing the logistic regression analyses relating sediment concentrations of aromatic hydrocarbons (AHs), polychlorinated biphenyls (PCBs), and chlorinated butadienes (CBDs) to prevalences of liver neoplasms in English sole in Puget Sound. The model (AHs + PCBs + fish size - CBDs - [AH + PCB interaction]) accounts for 35.3% of observed variation in neoplasm prevalence. An earlier version of this model was described by Malins et al. (1988).
% = Percentage variation in neoplasms prevalence explained; n.s.= effect not significant at $p>0.05$; * = effect significant at $p\leq0.01$; *** = effect significant at $p\leq0.0001$; **** = effect significant at $p<0.00001$.

sediment Σ PCBs. An antagonistic interaction between Σ AHs and Σ PCBs in sediment is also shown by this model. However, the fact that less than 35% of the variation in neoplasm prevalence can be explained by this model points to the importance of other factors, such as genetic differences, fish movement, and unidentified chemicals or their metabolites.

Significant correlations have been shown between levels of certain fluorescent aromatic compounds (FACs) and the prevalences of several types of idiopathic liver lesions in sole from multiple sampling sites in Puget Sound (Krahn et al., 1984; Krahn et al., 1986). Some FACs have been identified as metabolites of PAHs, including metabolites of B(a)P, pyrene, fluorene, dibenzofuran, phenanthrene, and fluoranthene (Krahn et al., 1987). Although the significant positive correlation between FAC levels and neoplasm prevalence has received most of the attention, equally strong and expected correlations exist between FAC levels and prevalences of megalocytic hepatosis and foci of cellular alteration (Table 3a), both of which are lesion types that have been directly implicated in the process of hepatic neoplasia in English sole (Myers et al., 1987). Fish from the same site have been shown to have no significant difference in the levels of bile FACs, regardless of whether the fish have or do not have significant idiopathic hepatic lesions (Table 3b). These data suggest that differences in FAC levels between fish are not caused by alterations in PAH metabolism, bile production, or other biliary function caused by the presence of hepatic lesions. Although in the original manuscript describing the differences in bile FAC levels between sole with and without idiopathic hepatic lesions, Krahn et al. (1984) reported a statistically significant difference ($p<0.05$) in levels of FACs fluorescing at B(a)P wavelengths in fish at a single contaminated site (the Duwamish Waterway), subsequent analyses of a larger

Table 3a. Spearman's rank correlation coefficients (r_S) and significance levels for prevalences of hepatic lesions and mean concentrations of bile metabolites (FACs) measured at benzo[a]pyrene wavelengths of English sole from sites in Puget Sound.

Lesion type	r_S	Significance level
Neoplasms	0.853	<0.002
Foci of cellular alteration (FCA)	0.773	<0.01
Megalocytic hepatosis (MH)	0.891	<0.001
Steatosis/hemosiderosis (Storage)	0.409	<0.5 (not significant)

Adapted from: Krahn et al., Archives of Environmental Contamination and Toxicology 1986;15:61-67.

Table 3b. Results of 2-way analysis of variance examining the effects of the presence (Yes) or absence (No) of idiopathic hepatic lesions on levels of fluorescent aromatic compounds (FACs) measured at benzo[a]pyrene wavelengths in bile of English sole, while controlling for the site of capture. Values shown are means expressed in ng/g. Values in parentheses refer to number of specimens in sample.

	Neoplasms		FCA		MH		Storage Disorders		Any Hepatic Lesion	
Site	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Everett	363	353	376	285	363	353	363	353	378	292
Harbor	(23)	(3)	(22)	(4)	(23)	(3)	(23)	(3)	(21)	(5)
Eagle	3019	2376	2829	3123	3183	2742	3049	2570	3558	2593
Harbor	(32)	(5)	(24)	(13)	(16)	(21)	(28)	(9)	(24)	(13)
Hylebos	942	680	882	1157	884	1058	880	1263	910	948
Waterway	(25)	(2)	(23)	(4)	(21)	(6)	(24)	(3)	(18)	(9)
Sitcum	771	1982	765	1766	855	1047	657	1995	618	1125
Waterway	(24)	(4)	(23)	(5)	(15)	(13)	(22)	(6)	(10)	(18)
Duwamish	560	543	526	600	552	563	519	619	516	574
Waterway	(25)	(7)	(19)	(13)	(20)	(12)	(20)	(12)	(10)	(22)
P-values for variables:										
Site	0.0002		0.0001		0.0001		0.0001		0.0001	
Lesion type	**0.2998**		**0.3867**		**0.9660**		**0.4861**		**0.7551**	

Table 4. Calculated odds ratios
for four categories of idiopathic h
site of collection are interpreted
Madison. Odds ratios for age rep
year of age on the odds of lesion

Lesion Type	Risk fac
Neoplasms	Upper Du
	Lower Du
	Age
Foci of Cellular	Upper Du
Alteration	Lower Du
	Age
Nuclear pleomorphism/	Upper Du
Megalocytic hepatosis	Lower Du
	North Se
	South Se
Steatosis/	Upper Du
Hemosiderosis	Age
	Winter se

Adapted from: L.D.Rhodes et al.,
407.

data set for sole from five polluted site
relationship, regardless of the lesion t
Consequently, FAC levels are properly
prevalences, and should be considered as

Table 4. Calculated odds ratios of significant ($p<0.05$) risk factors for four categories of idiopathic hepatic lesions. Odds ratios for the site of collection are interpreted relative to the reference site of Port Madison. Odds ratios for age represent the effect of each additional year of age on the odds of lesion occurrence.

Lesion Type	Risk factor	Odds Ratio
Neoplasms	Upper Duwamish Waterway	8.7
	Lower Duwamish Waterway	8.2
	Age	1.6
Foci of Cellular	Upper Duwamish Waterway	4.2
Alteration	Lower Duwamish Waterway	2.4
	Age	1.4
Nuclear pleomorphism/	Upper Duwamish Waterway	4.9
Megalocytic hepatosis	Lower Duwamish Waterway	3.4
	North Seattle Waterfront	4.0
	South Seattle Waterfront	2.7
Steatosis/	Upper Duwamish Waterway	2.9
Hemosiderosis	Age	1.4
	Winter season	0.001

Adapted from: L.D.Rhodes et al., Journal of Fish Biology 1987; 31:395-407.

data set for sole from five polluted sites in Puget Sound have failed to confirm this relationship, regardless of the lesion type considered in the analysis (Table 3b). Consequently, FAC levels are properly interpreted as being associated with lesion prevalences, and should be considered as an indicator of recent exposure to, uptake, and

metabolism of PAHs. However, because bile FAC levels measure recent uptake rather than long-term exposure to PAHs, and additionally, because levels in individuals may vary considerably due to differences in diet, reproductive status, excretion rate and other factors (Varanasi and Gmur, 1981; Collier and Varanasi, 1987), they are not necessarily an accurate predictor of the presence of specific hepatic lesions in individual fish.

Additional support for our hypothesis regarding the etiology of idiopathic lever lesions in English sole comes from a multiyear (1979-1984), epizootiologic study investigating the influence of certain potential risk factors on liver lesion prevalences in English sole, including fish age and gender, and site, season, and year of capture (Rhodes et al., 1987). The major results of this study are summarized in Table 4, showing odds ratios for particular risk factors as applied to the major liver lesion categories, with lesion prevalences at a non-urban reference station in Puget Sound (Port Madison) used as the baseline prevalences for calculation of the odds ratios for each potential risk factor. The results of this study show that certain risk factors inherent to the Duwamish Waterway (e.g. chemical contaminants) significantly and positively influence the prevalence of not only neoplasms, but also preneoplastic foci of cellular alteration, and the related degenerative conditions of nuclear pleomorphism and megalocytic hepatosis. Other less polluted urban sites in Elliot Bay (North Seattle waterfront, South Seattle waterfront) show elevated odds ratios only for nuclear pleomorphism and megalocytic hepatosis. Fish age emerges as a significant risk factor over the entire age range for all lesion categories shown in this table, with the exception of nuclear pleomorphism/megalocytic hepatosis. The formal epizootiologic approach used in this study (Rhodes et al., 1987) demonstrates: a) the insignificance of fish gender as a factor influencing the probability of idiopathic hepatic lesion development, b) the relative insignificance of season or year of capture as an influencing factor for most of the lesion types, c) the positive influence of increasing fish age, and therefore, increased time of exposure to contaminants, on the probability of developing preneoplastic and neoplastic hepatic lesions, d) the earliest age at which sole are affected with specific hepatic lesion types, and e) the significantly increased risk of developing certain idiopathic hepatic lesions for sole from polluted areas of Puget Sound.

INDUCTION OF HEPATIC LESIONS IN LABORATORY EXPOSURE STUDIES

The types of evidence discussed above are essentially associative and correlative, and do not constitute proof of a direct cause-and-effect relationship between exposure to chemical contaminants and tumour development in English sole. Long-term exposure studies designed to directly test the hypothesis that certain idiopathic liver lesions in wild English sole are the result of exposure to chemical contaminants in the sediment have recently been conducted in the laboratory.

The most significant results from these laboratory studies were the induction of nuclear pleomorphism, megalocytic hepatosis, and foci of cellular alteration in English sole after multiple intramuscular injections of either BaP, or a PAH-enriched fraction of a sediment extract from Eagle Harbor. These lesion types, which are important in the histogenesis of vertebrate hepatic neoplasms, were not observed in sham-injected, or solvent-injected controls, or in those fish injected with a fractionated extract from a relatively uncontaminated site in Puget Sound. Hepatocellular regeneration was detected at a significantly higher incidence, in comparison to the sham-injected controls, in both dosage

groups injected with sediment extract from Eagle Harbor (Schiewe et al., 1988; Varanasi et al., 1987b). The treatment protocol involved 13 injections of the sediment extracts or BaP, at four week intervals, followed by six months of holding without further treatment.

These lesion types temporally represent either the initial cytotoxic (nuclear pleomorphism and megalocytic hepatosis) and compensatory proliferative (hepatocellular regeneration) phases of hepatocarcinogenesis, or the consequent preneoplastic phase (eosinophilic and, primarily, basophilic foci). All of the lesions indicative of hepatotoxicity and compensatory proliferation, and the foci of cellular alteration were morphologically identical to those observed in wild English sole. The relationships among these lesions, in terms of their patterns of co-occurrence, were also essentially identical to those demonstrated in field studies in Eagle Harbor (Myers et al., 1987), with the exception that frank neoplasms were absent. Thus, the results from this laboratory exposure study directly implicate sediment-associated PAHs in the etiology of hepatocellular nuclear pleomorphism /megalocytic hepatosis, regeneration, preneoplastic foci of cellular alteration and, by association, hepatic neoplasms in English sole from Puget Sound. Moreover, in addition to the evidence discussed above supporting the view that liver neoplasms in wild English sole are the result of exposure to sediment-associated PAHs, other essential supportive evidence is based on the bioavailability and metabolism of PAHs, and DNA-PAH adduct formation in Puget Sound flatfish species (Varanasi et al., 1987a; Stein et al., this volume).

SUMMARY

The overall findings from our studies on the etiology of hepatic neoplasms and related disorders in English sole are supportive of a direct link between exposure to sediment-associated contaminants, mainly aromatic compounds, and the development of idiopathic hepatic lesions, including neoplasms. The major contributions to the establishment of this link are: a) the construction of a model of the histogenesis of hepatic neoplasia in English sole that parallels the same process in experimental hepatocarcinogenesis models, b) the development of a logistic regression model accounting for about 35% of the variation in neoplasm prevalence, c) the development of a separate logistic regression model showing increases in the probability of most idiopathic hepatic lesions occurring in sole from contaminated sites, and in older fish, d) the demonstration of significant correlations between levels of FACs in bile and prevalences of idiopathic hepatic lesions, and e) the experimental induction of unique hepatotoxic, regenerative, and putatively preneoplastic lesions in English sole by repeated exposure to a contaminated sediment extract.

In the case of hepatic neoplasia in wild English sole from Puget Sound, we have in essence a "natural laboratory" from which we can experimentally address the complex factors relating to etiology. Even though frank neoplasms were not induced in the laboratory, this is a unique hepatocarcinogenesis model, in that there are strong epizootiological data implicating environmental contaminants in the etiology of these lesions. This study is not affected by the problem inherent to other existing fish and rodent hepatocarcinogenesis models; that is, the inability to relate laboratory results directly to field observations. Notwithstanding these accomplishments toward the goal of defining the etiology of idiopathic hepatic disease in English sole, there is little information regarding the multiple genetic and epigenetic factors that influence, and the mechanisms and

processes that lead to, neoplasm development in fish. The importance of these variables is demonstrated in the logistic regression model of hepatic neoplasia in wild English sole, where the factors included in the model account for only 35% of the variation in neoplasm prevalence. Moreover, an increased understanding of additional risk factors, especially those that are promotional in nature, have the potential to strengthen the model. Development of such a "complete" model would be particularly advantageous in laboratory studies delineating the processes and mechanisms comprising the multistep pathway of hepatocarcinogenesis in fish.

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The Science of the Total Environment, 94 (1990) 51–69
Elsevier

OVERVIEW OF STUDIES ON LIVER CARCINOGENESIS IN ENGLISH SOLE FROM PUGET SOUND; EVIDENCE FOR A XENOBIOTIC CHEMICAL ETIOLOGY II: BIOCHEMICAL STUDIES

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ABSTRACT

The levels of aromatic hydrocarbons in sediments of Puget Sound, Washington, are positively correlated with the prevalence of hepatic neoplasms and related lesions in English sole (Parophrys vetulus). To investigate the biochemical processes involved in chemical carcinogenesis in fish from Puget Sound, we have studied the uptake, activation, and detoxication of polycyclic aromatic hydrocarbons (PAHs) in English sole, and have compared these data to PAH metabolism in a related species, starry flounder (Platichthys stellatus), which shows a lower prevalence of hepatic neoplasms than sole. The results of both laboratory and field studies show that sediment-associated PAHs are biologically available to both flatfish species, and that both species accumulate similar levels of PAHs. Analyses of hepatic DNA from sole using the ^{32}P-postlabeling technique indicate that xenobiotic chemicals were adducted to hepatic DNA of fish from the contaminated sites but not to the DNA of fish from reference sites. Studies of the ability of English sole and starry flounder to metabolize benzo(a)pyrene (BaP) and bind reactive BaP intermediates to hepatic DNA indicate that biochemical differences in the metabolism of carcinogenic PAHs may explain, at least in part, the apparent lower susceptibility of starry flounder than English sole to chemically induced hepatocarcinogenesis.

INTRODUCTION

Field studies in Puget Sound, Washington, USA, provide associational evidence that hepatic neoplasms in English sole (*Parophrys vetulus*) have a xenobiotic chemical etiology (Myers et al., this volume). Results from several studies have demonstrated significant positive correlations between levels of sediment-associated polycyclic aromatic hydrocarbons (PAHs) and prevalence of hepatic neoplasms, and thus implicate PAHs as major etiological agents. Further evidence for a PAH etiology of hepatic neoplasms in English sole was the recent induction of putatively preneoplastic basophilic foci in fish parenterally exposed to a PAH-enriched extract of a contaminated sediment from Puget Sound or to the carcinogenic PAH, benzo(a)pyrene (BaP) (Varanasi, 1987). The basophilic focus is

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considered to be a small carcinoma in the rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) hepatocarcinogenesis model (Hendricks et al., 1984). For many chemical carcinogens, such as PAHs, metabolism to reactive metabolites and their reaction with DNA appears to be a necessary step in the multistep process of chemical carcinogenesis.

A major focus of our research has been to gain an understanding of the processes of activation and detoxication of carcinogenic PAHs by English sole (Varanasi et al., 1987). Additionally, we have examined the bioavailability of sediment-associated PAHs to English sole, because sediments are major repositories of hydrophobic compounds, and bioavailability is an important issue in establishing cause-and-effect relationships between exposure to xenobiotic chemicals and observed biological abnormalities. We have also been interested in evaluating biochemical factors which may affect the initiation of chemical carcinogenesis in certain fish species. Thus, we are currently comparing the metabolism of BaP by English sole to that by starry flounder (*Platichthys stellatus*), a closely related species (Sakamoto, 1984) that exhibits a much lower prevalence of hepatic neoplasms when sampled from the same contaminated site (McCain et al., 1982).

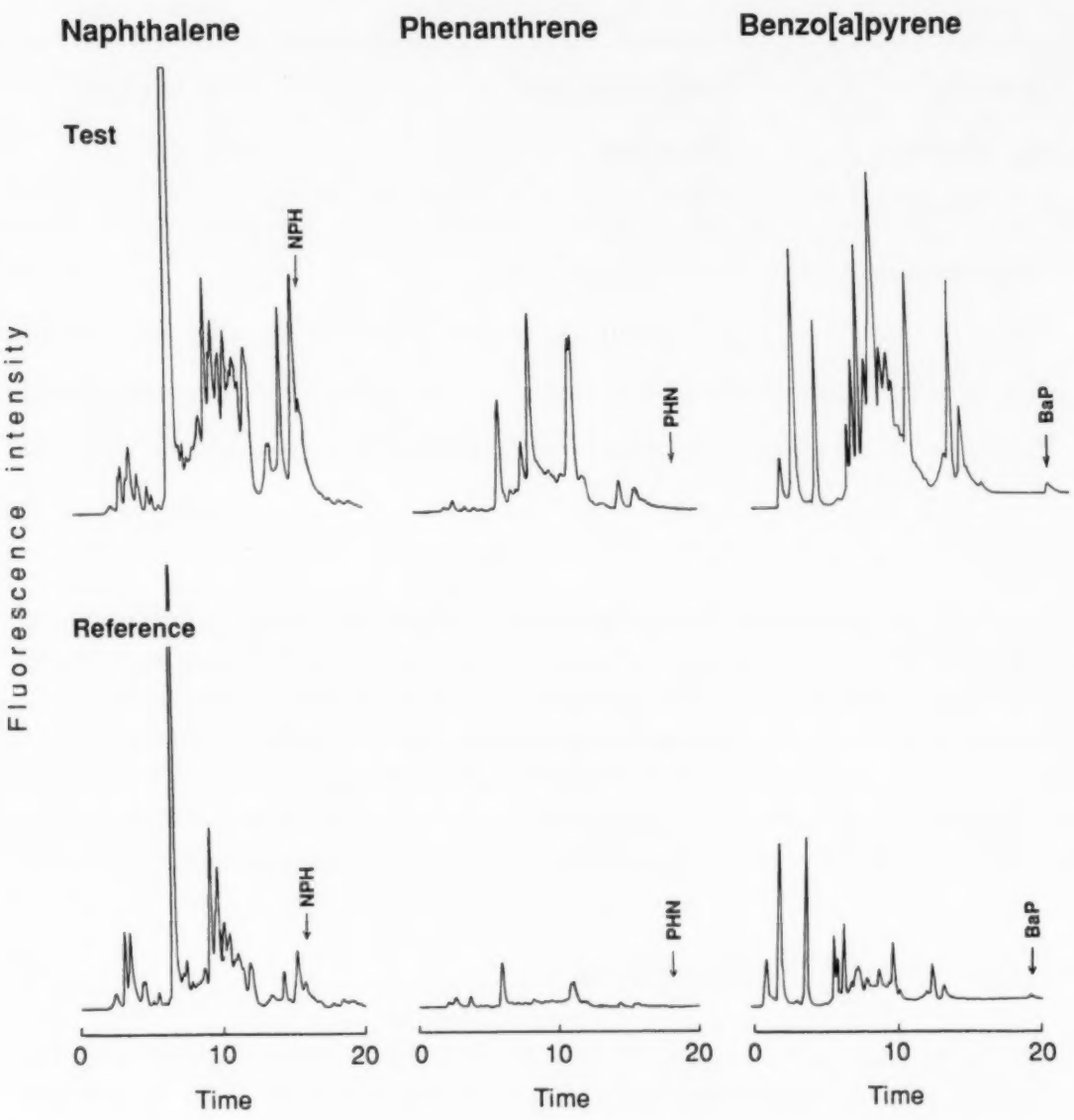
In this paper we will summarize our studies on the uptake and metabolism of PAHs by flatfish species of Puget Sound, as well as present recent findings from both field and laboratories studies on levels of DNA-xenobiotic adducts in liver of English sole (Varanasi et al., 1989a, Varanasi et al., 1989b) measured using the recently developed ^{32}P-postlabeling assay (Randerath et al., 1981). This assay has the advantage of not requiring the use of radiolabeled xenobiotic compounds, and combined with its high sensitivity, allows assessment of levels of DNA damage in wild fish sampled from sites contaminated with xenobiotic chemicals. Moreover, adducts of unknown structure can be detected with this method without the need of their full characterization. The ability to detect the formation and persistence of DNA-xenobiotic adducts in animals exposed to complex mixtures of chemicals is one of the most significant features of this method.

UPTAKE OF XENOBIOTIC CHEMICALS FROM SEDIMENT

The uptake of hydrophobic xenobiotic chemicals from sediment directly or indirectly may be the major source for accumulation of potentially carcinogenic compounds by benthic fish. Sediment may serve as a fairly direct source via desorption to interstitial water or by desorption of xenobiotic compounds from the sediment particles to gut epithelia after ingestion of sediment (McElroy et al., 1989). The uptake of sediment-associated chemicals by benthic invertebrates would serve as a more indirect route for the accumulation of xenobiotic compounds by benthic as well as pelagic fish which feed on such species. Recent analyses of stomach contents revealed that benthic invertebrates, such as small clams, amphipods and polychaetes, are major foods for English sole (P. Plesha, personal commun.).

Our studies (Stein et al., 1984, Stein et al., 1987; Reichert et al., 1985; Varanasi et al., 1985) using sediment from a contaminated site in Puget Sound, Washington, show that sediment serves as a direct source of hydrophobic xenobiotic chemicals to English sole, as well as to benthic clams (e.g., *Macoma nasuta*) and amphipods (e.g., *Rhepoxynius abronius*). In the study with English sole, fish were also exposed to sediment from a reference site that had similar physico-chemical characteristics (e.g., total organic carbon content) as the contaminated sediment but very low levels of PAHs or polychlorinated biphenyls (PCBs).

Using a recently developed HPLC/fluorimetric technique (Krahn et al., 1984, Krahn et al.,1986), analyses of bile, a major route of excretion of BaP metabolites by sole (Gmur and Varanasi, 1982; Stein et al., 1984), showed significantly higher (2 to 27 fold) concentrations of fluorescent aromatic compounds (FACs) in bile of fish exposed to the contaminated sediment than in those exposed to the reference sediment (Figure 1). Several of the FACs detected by this technique have been recently identified by gas chromatography/mass



1. Reversed-phase HPLC profiles of metabolites of fluorescent aromatic compounds (FACs) in bile of English sole exposed to test (Duwamish Waterway) and reference (Dosewallips River estuary) sediment for 88 days. The fluorescence of the HPLC eluate was measured at wavelength pairs (exitation/emission) for napthalene (NPH; 290/335 nm), phenanthrene (PHN; 256/380 nm and benzo(a)pyrene (BaP; 380/430 nm). Arrows indicate elution positions of the NPH, PHN, and BaP standards chromatographed under identical conditions. Adapted from Stein et al. (1987).

TABLE 1

Levels of fluorescent aromatic compounds in English sole and starry flounder from the Duwamish Waterway (contaminated site) and Saratoga Passage (reference site), Puget Sound, Washington.[a]

Species	Duwamish Waterway[b]	Saratoga Passage
English sole	690 ± 510 (12)*	70 ± 35 (12)
Starry flounder	980 ± 500 (12)*	200 ± 130 (12)

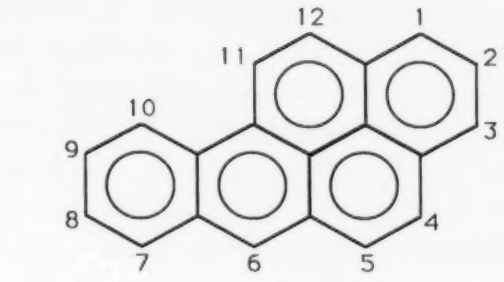
a Adapted from Collier and Varanasi 1987.

b Values (mean ± SD) are given as ng BaP-equivalents/ml bile. Asterisks denote significant ($p<0.05$) differences from values for fish from Saratoga Passage. No significant difference ($p>0.05$) between species was observed.

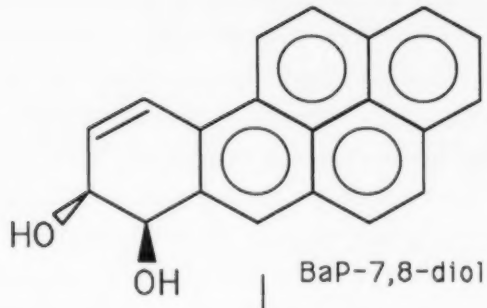
spectrometry as metabolites of 3-5 ring PAHs (Krahn et al., 1987). Additionally, a recent study (Collier and Varanasi, 1987) of the levels of FACs in bile of English sole and starry flounder from a contaminated and reference site in Puget Sound showed significantly higher FAC levels in both species from the contaminated site than in fish from the reference site (Table 1). Thus, these results implicate sediment as a major source of PAHs to English sole. However, at present the relative contribution of PAH-contaminated sediment and food organisms to the accumulation of these xenobiotic compounds by English sole is not known.

METABOLISM OF BENZO(A)PYRENE

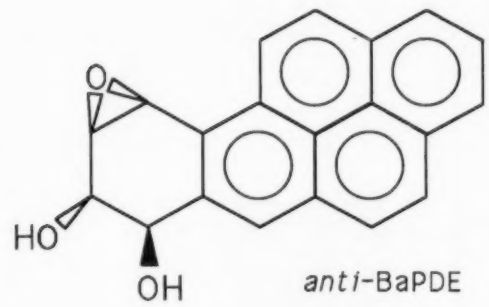
Biotransformation of PAHs, such as BaP, to electrophilic metabolites is a key step in the carcinogenic process (Miller and Miller, 1981; Guengerich, 1988). The liver of teleosts is generally the organ with the highest enzymatic activities for biotransformation of PAHs, which involves both oxidation and conjugation reactions (Bend and James, 1978; Stegeman, 1981; Buhler and Williams, 1989; Foureman, 1989). The first step in the oxidation of PAH by cytochrome P_{450} enzyme systems involves insertion of an oxygen atom to form an arene oxide. Arene oxides of PAHs are relatively unstable and can react further to yield glutathione conjugates, dihydrodihydroxy compounds (diols), or phenols, which can be further oxidized to quinones. Diols and phenols of PAHs are also substrates for conjugation reactions mediated by enzymes that catalyze glucuronidation and sulfation (James, 1986). Additionally, diols and phenols can undergo a second oxidation reaction to



BaP



BaP-7,8-diol



anti-BaPDE

2. The metabolic pathway for the formation of anti 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BaPDE) from benzo(a)pyrene (BaP). The BaP-7,9β-dihydrodiol is considered a necessary intermediate in the metabolic formation of anti-BaPDE.

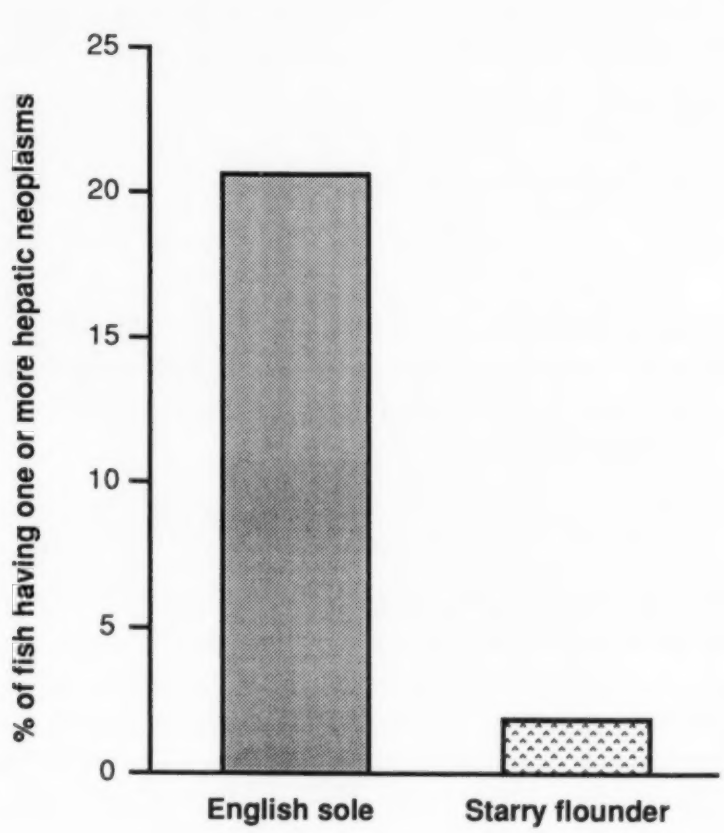
form diol-epoxides and phenol-oxides, some of which are believed to be the ultimate carcinogenic intermediates of PAHs and are known to interact covalently with DNA, RNA and protein (Sims and Grover, 1974; Osborne and Crosby, 1987). The suspected ultimate carcinogenic metabolite of BaP is (+)anti-BaP-7,8-dihydrodiol-9,10-oxide (anti-BaPDE), with (-)BaP-7,8-diol being the immediate precursor of anti-BaPDE (Figure 2).

Studies of the metabolism of BaP by English sole *in vivo* show that, regardless of the route of exposure, the hepatobiliary system accumulates the major proportion of BaP metabolites (Varanasi and Gmur, 1981; Stein et al., 1984; Varanasi et al., 1989c). In addition, studies using subcellular fractions of liver allow determination of specific pathways of metabolism and an assessment of the relative importance of individual enzymatic steps

in the balance between activation and detoxication. Therefore, studies using both *in vivo* and *in vitro* systems are necessary in delineating the metabolic pathways of PAHs in fish.

In Puget Sound, Washington, starry flounder from chemically polluted sites show a much lower prevalence of hepatic neoplasms than do English sole sampled from the same contaminated sites (Figure 3). A number of factors, singly or together, may account for these differences. One factor could be the differential accumulation of xenobiotic compounds. However, this does not appear to be the case, because English sole and starry flounder from a contaminated site where they show marked differences in prevalence of hepatic neoplasms, had similar levels of FACs in bile (Table 1.) These results are suggestive of similar bioavailability of PAHs to these species. Another factor could be differences in the activation and detoxication of xenobiotic compounds. To assess whether differences in the metabolism of PAHs may account at least in part for the differences in lesion prevalence, we have examined both the activation and detoxication of BaP, *in vitro* and *in vivo*, by these species and compared the results to those for rat, a species shown to be generally resistant to BaP-induced hepatocarcinogenesis (Langenbach et al., 1983).

The metabolism of BaP *in vitro* by hepatic microsomes of English sole and starry flounder shows formation of markedly higher proportions of bay-region diols (Varanasi et al., 1986), whereas, in rat, BaP-4,5-diol was the major diol formed *in vitro*. These results are similar to studies *in vivo* where the BaP-4,5-diol is the major conjugated diol detected in bile of rats



3. Prevalence of hepatic neoplasms in English sole and starry flounder from the industrialized Duwamish Waterway. Adapted from Krahn et al. (1986), and McCain et al. (1982).

TABLE 2

Metabolism of benzo(a)pyrene by liver of fish and rat.[a]

	AHH[b] Activity	BaP-7,8-diol[c] formation	DNA-BaP[d] binding in vivo
English sole	190±60	50±10 (26±3)[e]	28±5
Starry flounder	180±40	33±6 (22±2)	14±4
Sprague-Dawley rat	600±200	28±1 (6.7±1)	0.3±0.1

a Adapted from Varanasi et al. 1986; mean ± sem.

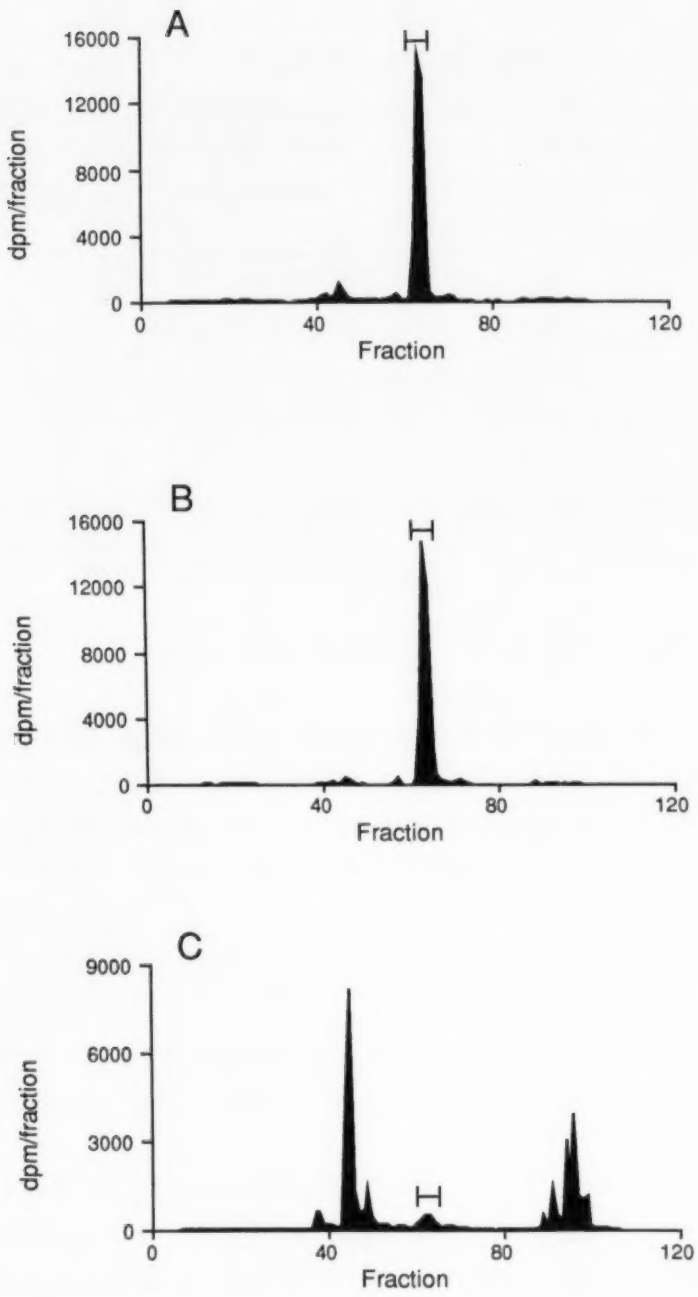
b Aryl hydrocarbon (BaP) hydroxylase, AHH (pmol BaP metabolized/min · mg protein), was measured at a substrate (BaP) concentration of 80 nmol/ml. Metabolites were separated from unmetabolized BaP by HPLC. All radioactivity eluting before the unmetabolized BaP was summed to determine amount of BaP metabolites formed. Hepatic microsomes from untreated fish and rat were prepared according to Collier et al. 1986.

c Rate of formation of the diol was determined from the same assays run to determine AHH.

d Fish (n=3-4) and rat (n=3) were administered 2.0 μg ^{3}H-BaP g^{-1} body weight intraperitoneally. The aquarium water temperature was 14±1°C. Livers were sampled 24h later and the DNA isolated according to Varanasi et al. 1986. The level of binding of BaP-derived radioactivity is expressed as pmol BaP equivalents /mg DNA.

e The values in parentheses are the percent BaP-7,8-diol of the total BaP metabolites.

(Chipman et al., 1981), and BaP-7,8-diol is the major conjugated diol in bile of fish (Gmur and Varanasi, 1982; Varanasi et al., 1986). Additionally, it was shown that English sole and starry flounder metabolized BaP at a rate that was only about 30% of the rate of BaP metabolism by rat (Table 2). The rate of formation of BaP-7,8-diol, however, was similar



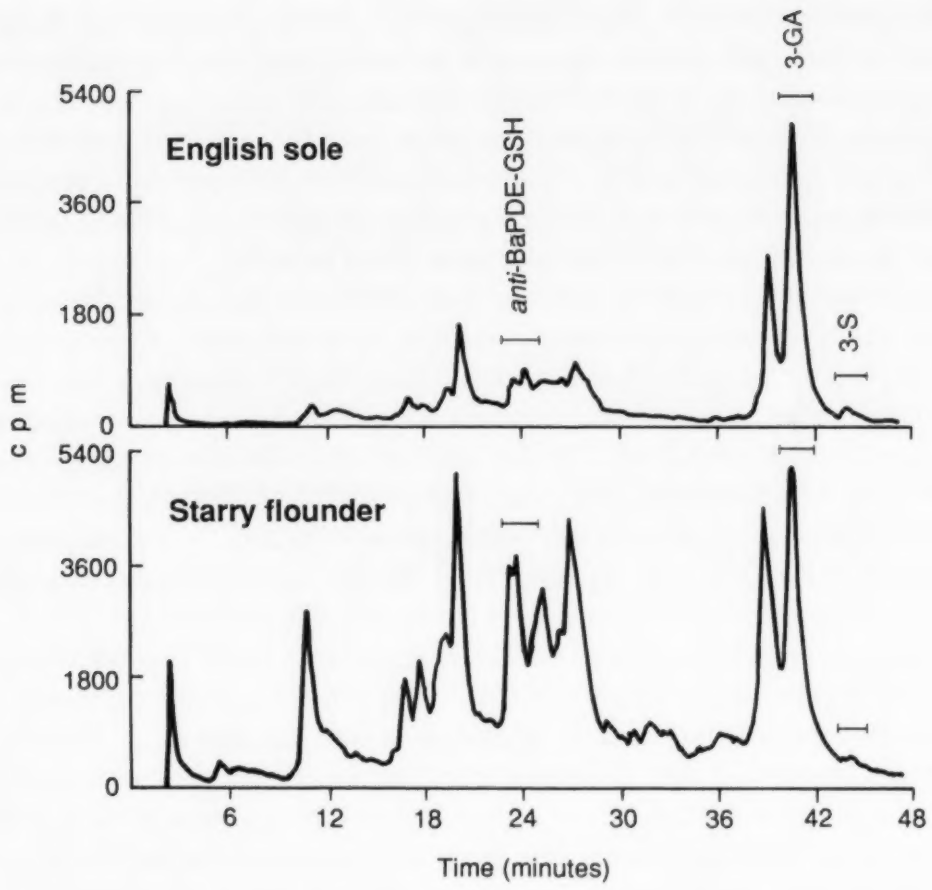
4. Reversed-phase HPLC profiles of [^{3}H]benzo(a)pyrene metabolite-deoxyribonucleoside adducts formed by hepatic microsomes of (A) English sole (B) starry flounder and (C) Sprague Dawley rat. Salmon testes DNA which had been modifed in a binding assay was purified and hydrolyzed with DNase I, snake venom phosphodiesterase, and alkaline phosphatase to mononucleosides. Modified adducts were isolated by Sephadex LH-20 column chromatography and analyzed by reversed-phase HPLC using an Altex Ultrasphere ODS column. The bars represent where the internal standard of [^{14}C]-anti-BaPDE-deoxyguanosine adduct chromatographs. Adapted from Nishimoto and Varanasi (1985).

among all species (Table 2). Thus, the proportion of BaP converted into the penultimate carcinogen of BaP, BaP-7,8-diol, was similar for both fish species but was more than three times as great as that in rat (Table 2), and thus provides some evidence for the potential tumorigenicity of PAHs, such as BaP, in these wild fish. For several PAHs, reactive metabolites can covalently bind at several sites on DNA. However, in many cases only one adduct shows a correlation with tumor formation (Dipple et al., 1984). Therefore, it is important to identify and quantitate individual DNA adducts.

Determination of the types of DNA adducts formed *in vitro* using hepatic microsomes of fish and rat revealed differences in BaP-DNA adduct profiles. Reversed-phase HPLC analysis of [^{3}H]BaP-modified deoxyribonucleosides from incubations with salmon sperm DNA and hepatic microsomes from control juvenile English sole and starry flounder (Figure 4) showed a single major adduct with the identical retention time as the (+)anti-BaPDE-deoxyguanosine standard (Nishimoto and Varanasi, 1985; Nishimoto and Varanasi, 1986; Nishimoto, 1986). As mentioned previously, (+)anti-BaPDE is the suspected ultimate carcinogen of BaP (Figure 2). In contrast to the fish species, incubations using hepatic microsomes from rat (Figure 4) showed that the 9-hydroxy-BaP-4,5-oxide-deoxyguanosine was the major adduct, whereas, the BaPDE adduct was a minor product (Varanasi et al., 1987). These results are consistent with BaP-7,8-diol being a major metabolite formed by liver of the fish species and a minor metabolite in rat (Varanasi et al., 1989d).

The results in Table 2 also show species differences in the level of *in vivo* binding of [^{3}H]BaP to hepatic DNA. Total DNA adduct levels in the fish were 47 to 93 times as great as the level for rat, when all species were given equimolar doses of BaP i.p. (Table 2). The findings that rat liver converts only a small proportion of BaP to BaP-7,8-diol and that the anti-BPDE-DNA adduct was a minor product may contribute to the low level of binding of BaP to rat liver DNA *in vivo* compared to English sole and starry flounder liver DNA. Furthermore, differences in rate of removal of BaP-DNA adducts may also be an important factor in the differences in binding between fish and rat. In rat, hepatic BaP-DNA lesions are effectively repaired (Friedberg and Hanawalt, 1988), whereas in English sole, a significant decline in the level of adducts was not observed until four weeks after treatment with [^{3}H]BaP (Varanasi et al., 1987).

Although the extent of formation of BaP-7,8-diol and the repair of DNA lesions may explain the differences in DNA binding levels between fish and rat, the similarity between English sole and starry flounder in the proportion of BaP converted to BaP-7,8-diol *in vitro* is not consistent with the two-fold lower level of hepatic BaP-DNA adducts in starry flounder compared to English sole (Table 2). Clearly, other factors may also contribute to the observed differences in the binding of BaP metabolites to hepatic DNA between these fish species. One possible explanation may be species differences in the rates of detoxication of reactive BaP metabolites that bind to DNA. A major pathway for detoxication of reactive PAH metabolites involves the hepatic glutathione-S-transferases (GSTs), which catalyze the reaction between oxides, such as BaPDE, and the tripeptide, glutathione (GSH) to yield a thioether conjugate, thereby decreasing the level of reactive metabolite available to bind to DNA (Ketterer et al., 1983). Recently, Plakunov et al. (1987) developed an ion-pair reversed-phase HPLC technique that separates the BaP-thioether conjugates from other BaP conjugates (e.g. glucuronides and sulfates). We have adapted this technique for the analysis of fish bile (Nishimoto, 1986; Varanasi et al., 1989c). The chromatograms in Figure 5 show that for starry flounder exposed to [^{3}H]BaP, a greater



5. Ion-pair reversed-phase HPLC separation of conjugated benzo(a)pyrene (BaP) metabolites present in bile of English sole and starry flounder exposed to 2.0 μg [^{3}H]BaP g^{-1} for 24 hours. Internal standards of anti-BaP-sulfate (3-S) were added to each sample prior to chromatography. The HPLC eluant was monitored by both fluorescence and UV detectors, and radioactivity in the eluant was determined every 0.2 minutes using a Radiomatic Flow-one detector. Adapted from Nishimoto (1986).

proportion of BaP-derived radioactivity eluted in the region of the chromatogram where the BaPDE-GSH standard eluted, than observed for English sole exposed to the same dose of BaP. Concomitantly, the proportion of BaP-derived radioactivity present as BaP-7,8-diol glucuronide in bile of starry flounder (11 ±1%) was significantly less than the proportion in bile of English sole (14 ± 1%) (Varanasi et al., 1986). Thus, this finding, in addition to the results showing no differences in the rate of formation *in vitro* of BaP-7,8- diol between species, suggest greater conjugation of reactive BaP metabolites with GSH in starry flounder relative to English sole. In support of these findings, Collier (1988) has found that, regardless of site of capture, starry flounder consistently have nearly a three-fold higher GST activity in liver than does English sole, as measured using 1-chloro-2,4-dinitrobenzene as the substrate. Studies with rat hepatocytes show that the level of hepatic GST activity is negatively correlated with the level of BaP-DNA binding (Jernstrom et al., 1982).

Overall, these results suggest that the observed lower level of binding of [^{3}H]BaP to hepatic DNA in starry flounder than English sole *in vivo* is the result of fewer reactive

BaP metabolites being available for reaction with DNA, possibly as a consequence of their conjugation with GSH. These biochemical differences in the metabolism of BaP, a carcinogenic PAH, may partially explain the apparent lower susceptibility of starry flounder than English sole to chemically induced hepatocarcinogenesis. To further support this conjecture, additional studies in both species are needed to: a) identify further the adducts of BaP metabolites with both GSH and hepatic DNA and assess the dose-response for formation of both, b) characterize species differences in the GST enzyme profiles and the substrate specificities of the individual enzymes, and c) determine the relative ability of each species to repair PAH-induced DNA damage. A better understanding of the species differences in the level of BaP modification of hepatic DNA should emerge from such studies. Moreover, because these and other fish species are exposed to myriad PAHs in their environment and may have complex DNA adduct profiles, methods with the ability to detect all adducts present in fish from field studies as well as laboratory experiments using complex environmental mixtures are especially needed.

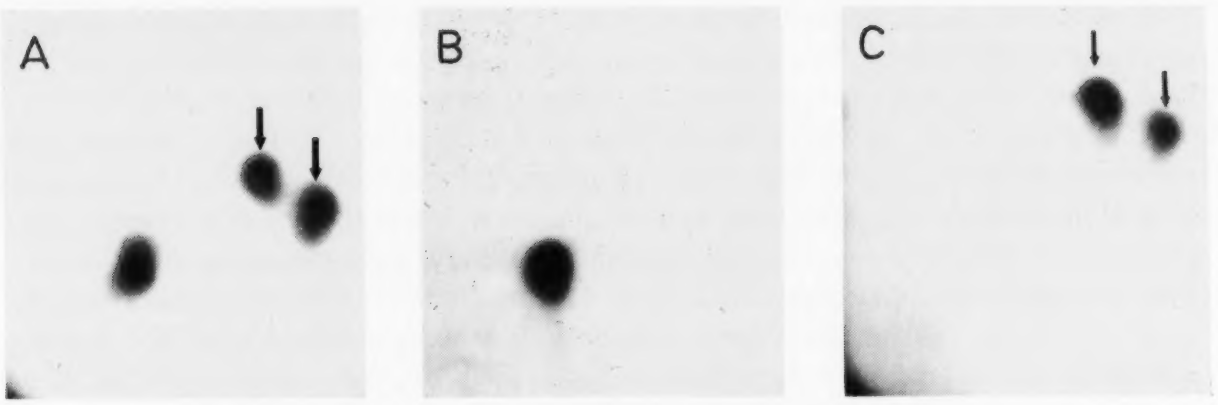
32P-POSTLABELING ANALYSIS OF DNA ADDUCTS

Studies of covalent carcinogen-DNA interactions *in vivo* often use either radiolabeled carcinogens, fluorescence spectroscopy, immunoassays, or ^{32}P-postlabeling to detect and quantitate the formation of DNA adducts. Each technique has its advantages and disadvantages. However, central to the choice of method is sensitivity of the assay and its applicability to animals exposed to complex mixtures.

At present, the ^{32}P-postlabeling assay offers the lowest limit of detection (1 adduct in 10^9 -10^{10} nucleotides) and does not require characterization of individual adducts before they are measured. There are several versions of the ^{32}P-postlabeling assay (Gupta and Randerath, 1988), and we have used the version that separates xenobiotic-modified nucleotides from normal nucleotides by n-butanol extraction (Gupta, 1985). This increases the sensitivity of the assay by allowing analysis of larger quantities of DNA. The remaining normal nucleotides and adducts are separated by anion-exchange TLC on polyethyleneimine (PEI) cellulose sheets, and the ^{32}P-labeled adducts are detected by autoradiography. The TLC maps usually exhibit characteristic ^{32}P-labeled spot patterns not seen on maps of appropriate controls.

Laboratory Studies

A study by Varanasi et al. (1989a), using the ^{32}P-postlabeling assay was conducted to determine the pattern of DNA adducts formed in BaP-exposed English sole *in vivo*. The autoradiogram of a ^{32}P-labeled hepatic DNA digest from an English sole treated with 15 $\mu g\ g^{-1}$ of BaP (Figure 6a) showed a single major adduct that had the same chromatographic characteristics as the major adducts present in DNA incubated with anti-BaPDE standard (Figure 6b). This ^{32}P-labeled spot was not evident in autoradiograms of hepatic DNA digests of control English sole (Figures 6c). Additionally, these studies revealed a linear ($r = 0.996$) response in the formation of the anti-BaPDE-DNA adduct at doses of BaP from 2 to 100 $\mu g\ g^{-1}$ (Varanasi et al., 1989a). These results suggest that in the dose range investigated, neither metabolic activation nor detoxication pathways were saturated.



6. Autoradiograms of PEI-cellulose thin-layer chromatograms of ^{32}P-labeled digests of hepatic DNA from English sole exposed to a dosage of 15 μg BaP g^{-1} (A) or to vehicle only (C) for 24 hours. In addition, the autoradiogram of the standard anti-BaPDE bound to salmon testes DNA (B) is given to show the chromatographic properties of the anti-BaPDE-deoxyguanosine-3',5'-bisphosphate. The D1 development was run in 2 M lithium chloride. The D3 development, bottom to top, was in 4.5 M lithium formate, 9.3 M urea, pH 3.5; and the D4 development, at a right angle to D3, was run in 1.6 M lithium chloride, 0.5 M Tris, 9.3 M urea, pH 8.0. The D5 development, same direction as D4, was done using 1.6 M sodium phosphate, pH 6.0, with a 10 cm Whatman chromatography paper wick attached to the top of the sheet. The spots indicated by arrows are present in nearly all DNA samples and can be considered background. The origin is located at the bottom left-hand corner of each TLC map. Additional information and methodology can be found in Varanasi et al., (1989a).

Further, at a dosage of 15 μg g^{-1}, which is similar to that shown to produce putative preneoplastic lesions in English sole (Varanasi, 1987), no significant decline in the level of the anti-BaPDE adduct was observed for up to 60 d post-exposure (Varanasi et al., 1989a). This persistence of the anti-BaPDE-DNA adduct is consistent with low levels of unscheduled DNA synthesis in fish cell lines compared to mammalian cell lines when the cells are exposed to a genotoxic agent (Walton et al., 1983). The persistence of BaPDE-DNA adducts in sole suggests that hepatic DNA adducts may serve as molecular dosimeters of the cumulative exposure of fish populations to genotoxic compounds.

The level of specific DNA-adducts in target tissues are regarded as indicators of the carcinogenic potential of a compound (Lutz, 1979). For example, the tumorigenic activity of BaP and level of anti-BaPDE-DNA adducts in mouse epidermal DNA have nearly parallel dose-response curves (Ashurst et al., 1983). The persistence of the anti-BaPDE-DNA in English sole would be expected to lead to an accumulation of significant levels of anti-BaPDE-DNA adduct during chronic exposure to BaP. Moreover, the persistence of the bulky anti- BaPDE-DNA adduct may lead to interference in the fidelity of DNA replication (Stowers and Anderson, 1985). These considerations point out the importance of conducting experiments on the effects of chronic exposure to contaminants on the formation and repair of DNA adducts in fish, because this situation is encountered both during environmental exposures and in many experimental

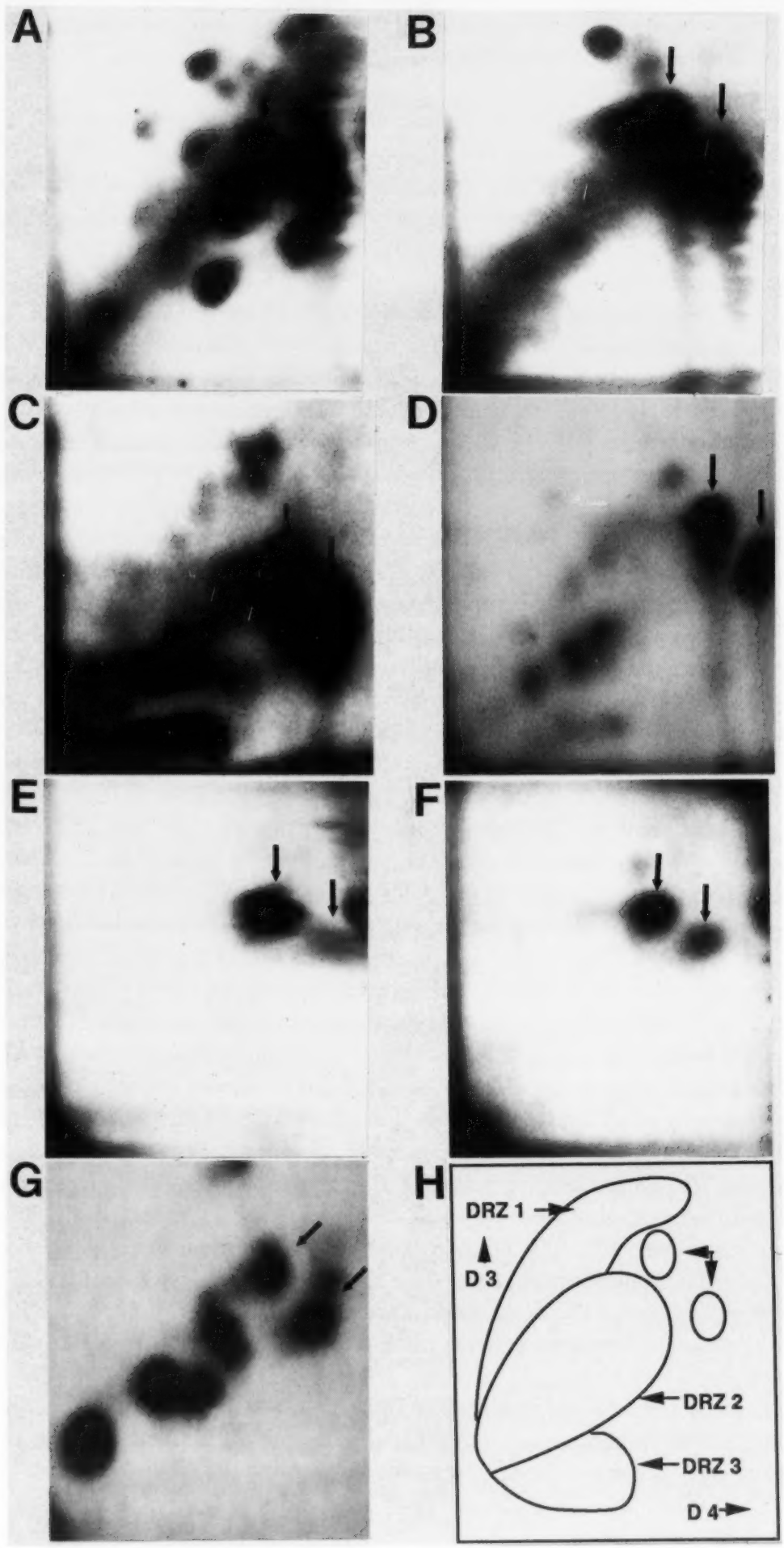
tumorigenesis studies. The ^{32}P-postlabeling assay is particularly well-suited to determine the levels of xenobiotic-DNA adducts formed during such experiments.

Field Studies

We have recently detected the presence of hepatic DNA adducts in English sole (Varanasi et al., 1989 b) from the Duwamish Waterway and Eagle Harbor, Puget Sound, Washington. English sole from these sites exhibit an elevated prevalence of hepatic neoplasms, and are exposed to significant levels of xenobiotic compounds in the sediments (Myers et al., this volume). The profiles of DNA adducts in fish from these sites were compared to those for fish from a reference area (Polnell Point) and to fish from a reference area exposed to organic extracts of sediments from the two contaminated sites and the reference site. The Duwamish Waterway sediment extract (DWSE) and Eagle Harbor sediment extract (EHSE) were prepared using the same batches of sediments and extraction procedures of Collier et al. (1986) and Varanasi et al. (1987), respectively. The Polnell Point sediment extract (PPSE) was recently prepared using the same procedure as used for the EHSE sample. English sole were exposed to the extracts so that qualitative comparisons could be made with fish sampled from the respective sites. Therefore, only qualitative information on the composition of the extracts will be provided here. Both contaminated sediments contained PAHs having 2 to 6 benzenoid rings, whereas these compounds were either nondetectable or present at very low concentrations in the reference sediment extract. PCBs were the major chlorinated compounds present in the DWSE and were virtually absent from the EHSE and PPSE. Quantitative information on the levels of PAHs and chlorinated hydrocarbons for the contaminated sediments is presented in Collier et al. (1986) and Varanasi et al. (1987).

The autoradiograms of ^{32}P-labeled hepatic DNA digests from English sole from the contaminated sites exhibited up to three diagonal radioactive zones (DRZs) radiating from the origin, which were not present in autoradiograms for fish from the reference site (Figure 7). The DRZs contained distinct spots, as well as what appeared to be multiple overlapping adduct spots (Figure 7a and Figure 7c). Autoradiograms of TLC maps of DNA adducts for all English sole from the contaminated sites consistently exhibited DRZ-2. The DNA adducts of the PAHs benzo(a)pyrene, chrysene and dibenz(a,h)anthracene, formed *in vitro* using English sole hepatic microsomes, chromatographed in the region of the TLC maps designated DRZ-2 (Figure 7g). Moreover, DRZ-2 was present in the autoradiograms of ^{32}P-labeled hepatic DNA from English sole treated with EHSE and DWSE (Figure 7b and Figure 7d), but was not present in TLC maps of ^{32}P-labeled DNA from fish treated with PPSE (Figure 7f). The autoradiograms of TLC maps for fish from Eagle Harbor and those exposed to EHSE also consistently showed additional DRZs, such as DRZ-1 and DRZ-3 (Figure 7c and Figure 7d). The DRZ-3 was consistently present in all autoradiograms of DNA of fish from Eagle Harbor, whereas the level of adducts in DRZ-1 varied to a greater extent among individual fish from Eagle Harbor. Moreover, DRZ-1 and DRZ-3 were consistently present in autoradiograms of ^{32}P-labeled DNA digests of EHSE-treated fish (Figure 7d).

Overall, these results show that hepatic DNA of English sole from contaminated sites where they exhibit a high prevalence of hepatic neoplasms, contained a suite of adducts indicative of exposure to genotoxic compounds. The chromatographic characteristics of the


A
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adducts and similarities in adduct profiles between wild fish and those treated with contaminated sediment extracts suggested that hydrophobic aromatic compounds of anthropogenic origin were adducted to hepatic DNA of fish from the contaminated sites but were not bound to DNA of fish from the reference site. Furthermore, the finding that carcinogenic PAHs yielded DNA adducts that chromatographed in the region of the TLC maps designated DRZ-2, suggested that a portion of the adducts in hepatic DNA of English sole from the contaminated sites may be due to the covalent binding of PAH metabolites to DNA. Recent ^{32}P-postlabeling analyses of hepatic DNA from brown bullhead (*Ictularus nebulosus*) from the PAH-contaminated Buffalo and Detroit Rivers also revealed elevated levels of adducts in the area of the autoradiogram we have designated DRZ-2, as compared to adduct levels in aquaria-raised bullhead (Dunn et al., 1987). Studies are currently in progress to examine species differences in hepatic DNA adduct formation among flatfish species (e.g., English sole, starry flounder, and rock sole, *Lepidopsetta bilineata*) from several contaminated and reference sites in Puget Sound.

PROSPECTUS

Presently, much of the evidence supporting the hypothesis of a chemical etiology for hepatic neoplasms in English sole and other fish species comes from the association of high levels of anthropogenic chemicals in sediments with high prevalences of liver neoplasms. This type of correlation requires many assumptions to be made regarding the bioavailability of the sediment-associated chemicals and their metabolism and disposition in the fish. By using the ^{32}P-postlabeling assay to determine the level of DNA adducts present in target tissues, the level of exposure of fish to xenobiotic chemicals can be expressed in terms of the amount of chemical reaching a critical target site, DNA, and thereby integrates the pharmacokinetic factors such as biovailability, tissue distribution, metabolism, and detoxication. This suggests that the ^{32}P-postlabeling assay will be a useful tool in delineating further the etiology of hepatic neoplasia in benthic fish species from PAH-contaminated sites, as well as allow the use of DNA adducts as a molecular dosimeter of

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7. Autoradiograms of PEI-cellulose thin-layer chromatograms of ^{32}P-labeled digests of hepatic DNA from English sole caught from contaminated sites (A: Duwamish Waterway; B: Eagle Harbor) and reference sites (C: Polnell Point) of Puget Sound, Washington. In addition, autoradiograms are given of ^{32}P-labeled digests of hepatic DNA from English sole exposed to extracts of sediments from Duwamish Waterway (D), Eagle Harbor (E), and Polnell Point (F). A composite autoradiogram of DNA adducts formed from the metabolism by English sole hepatic microsomes of BaP, chrysene and dibenz[a,h]anthracene (G) is given to show the chromatographic properties of ^{32}P-labeled DNA adducts of 4-5 ring aromatic hydrocarbons. The regions of diagonal radioactive zones (DRZs) 1-3 are outlined in chart H. The solvent systems used for the separation of ^{32}P-labeled DNA adducts were the same as described in Figure 6. The spots indicated by arrows are present in nearly all DNA samples and can be considered background. The origin is located at the bottom left-hand corner of each TLC map. Additional information and methodology can be found in Varanasi et al., (1989b).

the exposure of aquatic species to complex mixtures of potentially genotoxic compounds. However, this is not to suggest that this assay is a panacea for our studies of chemical carcinogenesis in wild fish. The differences observed in the DNA adduct profiles of English sole from the two contaminated sites alert us to yet unidentified genotoxic chemicals bound to hepatic DNA of this species. Thus, the multiplicity of DNA adducts in wild English sole emphasizes the complexities and difficulties to be faced in identifying individual adducts in organisms continuously processing myriad chemicals.

ACKNOWLEDGEMENTS

We are grateful to Ms. Bich-Thuy Le Eberhart, Mr. Tom Hom, and Mr. Herb R. Sanborn for their excellent support with our studies cited in this review, and to Dr. Tracy K. Collier and Mr. Mark S. Myers for their timely review of this manuscript. These studies were partially supported by the Office of Oceanograhpy and Marine Assessment, NOAA.

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The Science of the Total Environment, 94 (1990) 71–87
Elsevier

RELATIONSHIP BETWEEN LIVER TUMORS AND AGE IN BROWN BULLHEAD POPULATIONS FROM TWO LAKE ERIE TRIBUTARIES

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ABSTRACT

We compared liver tumor frequencies, and age and length characteristics of brown bullheads (Ictalurus nebulosus) of greater than 250 mm total length from two Lake Erie tributaries. Bullheads taken from Old Woman Creek (n=144) had no grossly observable liver tumors, while those collected in the highly industrialized Black River (n=532) had a 30% frequency of grossly visible liver tumors during 1981-1982. Liver lesions diagnosed histologically in a randomly collected sample (n=125) of brown bullheads from the Black River included both biliary and hepatic lesions, with cancerous neoplasms occuring in 38.4% of the fish. Black River bullheads of combined ages 4 and 5 had a significantly ($p \leq 0.05$) greater prevalence of biliary carcinomas (35.5%) than those of ages 2 and 3 combined (18.4%). Biliary carcinoma was significantly more prevalent than hepatocellular carcinoma in age 4 fish (sexes combined) and in males of ages 3 and 4. The prevalence of hepatocellular carcinoma was significantly higher in females than in males. Age distributions of bullheads differed significantly between the two sites, while length distributions were similar. No brown bullheads of ages 6 or 7 were collected in the Black River, while these age groups composed 18% of the catch in Old Woman Creek. Brown bullheads of age 5 were almost six times more numerous in the Old Woman Creek than in Black River collections. These age and length distributions are consistent with the hypothesis that brown bullheads in the Black River were subjected to an age-selective mortality associated with high prevalences of liver carcinoma.

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INTRODUCTION

A strikingly high prevalence of liver neoplasia, based on gross observations, was reported in brown bullheads (*Ictalurus nebulosus*) from the Black River, Ohio (Baumann et al., 1987). All of the grossly observable lesions were diagnosed in subsequent histological examination as biliary in origin, but some hepatic neoplasms were observed in adjacent tissues. These observations suggested that the frequency of neoplasia was being underestimated by gross observations. Therefore, a more intensive histopathological survey was conducted to better characterize these liver neoplasms and to more accurately estimate their occurrence. Preliminary results verified a high frequency of hepatocellular tumors, as well as cholangiocellular tumors, in brown bullheads of ages 3 and older from the Black River (Baumann and Harshbarger, 1985). In this study, we describe the distribution of these neoplasms by age group and sex of the fish. We also compare the age composition and lengths of brown bullheads from the Black River and a reference site (Old Woman Creek), with the intention of determining whether high prevalences of tumors have an effect upon the structure of bullhead populations.

METHODS

Sampling Locations and Field Collections

Brown bullheads were collected from two Lake Erie tributaries in Ohio; the Black River at Lorain, and Old Woman Creek at Huron (Figure 1). At a point near the collecting site, the Black River received effluents from a steel plant and associated coking facility during the study period (1981 and 1982). Old Woman Creek, about 32 km west of the Black River, has a small, primarily agricultural, watershed. Its lower reach, which includes the collecting site, is part of the Old Woman Creek National Estuarine Sanctuary.

Bullheads were collected by overnight sets of fyke nets, with either 12 mm or 25 mm stretched mesh and 9 m leads. Fish of less than 250 mm total length were discarded to exclude fish of age 2 or less from the sample, since the grossly observable tumors reported by Baumann et al. (1987) occurred in less than 2% of the livers of 2 year-old fish.

Fish were collected from the Black River in 1981 and 1982, and from Old Woman Creek during the period 1984 to 1986. For comparison of age, size, and sex ratios, we pooled data by season within collection sites; that is, spring (April-May) and late summer and fall (August-November). Histopathological examinations were performed on random samples of livers from 125 brown bullheads taken from the Black River in May, 1982, and from 10 bullheads taken from Old Woman Creek in August, 1984.

Necropsy and Histology

Each specimen was inspected for grossly observable lesions involving the skin, the gills, or other tissues within the gill cavity, and tissues within the oral cavity. The abdomen was incised from the anus to the isthmus and the body cavity was manually spread for inspection of the viscera. Unusual features that were grossly visible were noted, and livers with tumor-like abnormalities were often photographed. For the histopathological survey, the

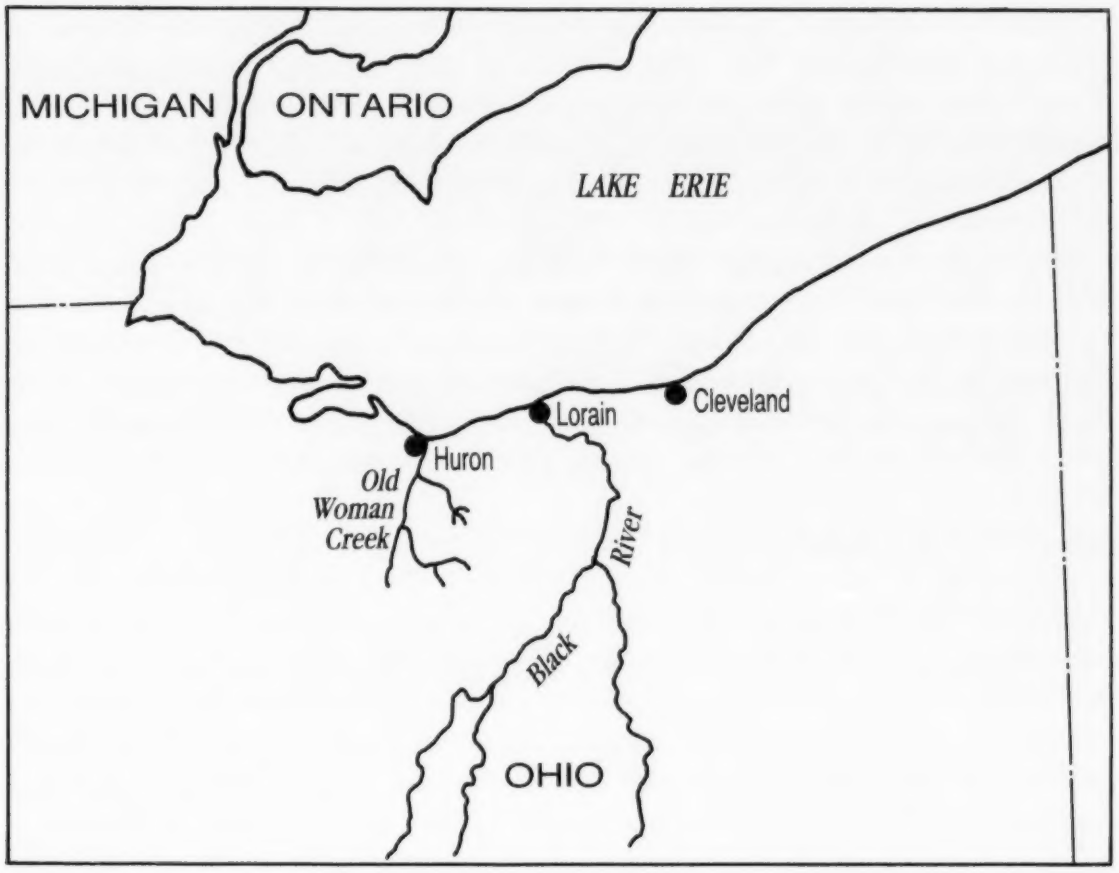

Figure 1: Locations of the Black River and Old Woman Creek in the Lake Erie drainage basin.

liver and all gross lesions within surrounding tissues were excised and immediately preserved in 10% neutral buffered formalin. Only the results of the liver histopathology are reported here.

Tissue blocks were cut from four to seven levels, depending on the size of the organ. These blocks were then dehydrated with an alcohol series up to absolute, infiltrated with paraplast, embedded, and sectioned at 5 μm. Tissue sections were routinely processed and stained with hematoxylin and eosin. Liver sections were examined by light microscopy and the lesions were diagnosed. Tissue slides from all livers examined are archived in the Registry of Tumors in Lower Animals, Smithsonian Institution.

Aging

Pectoral spines were collected in the field and processed in the laboratory using a modification of the methods of Marzolf (1955) and Scholl (1968). Spines were placed into individual 20 mL scintillation vials and decalcified with 5% aqueous nitric acid. Decalcification required 14-18 h, depending on the thickness of the spine. The process was halted when spines were flexible but not limp. Nitric acid was then replaced by 50-60% ispopropyl alcohol for storage.

A 6 to 10 mm section of spine just anterior to the basal groove was removed with a single-edge razor blade. This spine section was then mounted on a cryostat palat with Tissue Tek-II[1] frozen embedding medium. The spine was trimmed and sectioned using an International-Harris cryostat model CT[1], and the sections were returned to the alcohol solution. Taking sections of different thicknesses (25-150 μm) increased the probability of having some that were readable.

Dehydration was accomplished by processing a section through a series of 25%, 50%, and 100% glycerol solutions on a standard microscope slide covered with a 22 x 50 mm coverslip and sealed with clear nail polish. Both a dissecting scope with refracted light and an Eberbach[1] projection scope commonly used for reading scales, were used to read the spines. The projection scope provided superior resolution and facilitated simultaneous examination by two or more persons when the age was difficult to determine.

Categorization and Statistics

Individual fish were categorized according to the most advanced liver lesion diagnosed histologically. Thus, a fish that had both a cancerous, and a noncancerous or precancerous lesion would be included with the segment of the population with cancers. However, a fish could be enumerated for both biliary and hepatic neoplasms, if the fish contained both types. A Chi-square test was used to statistically compare categories of age, sex, and tumor frequency. The level of significance was set at $p \leq 0.05$, unless otherwise indicated.

RESULTS

Liver Tumor Diagnosis

Neoplasms or incipient neoplasms of both hepatocytic and cholangiocytic origin were diagnosed in livers of brown bullheads from the Black River. Neither type, regardless of size or stage, was encapsulated or circumscribed. Metastases were not seen, even though tumors of both cell types have metastatic capability in brown bullheads, based on observations of bullhead specimens in the of Registry of Tumors in Lower Animals (accession numbers 3782 and 3785, contributed by Marilyn J. Wolfe), and observations of bullheads collected in the Cuyahoga River (unpublished data). Both types of neoplasms showed progression from subtle changes to obvious cancer, without clearly defined intermediate stages. These observations suggest that the most prudent course might be to call them all either hepatocytic neoplasms or cholangiocytic neoplasms, followed by the appropriate modifiers. However, to be consistent with mammalian studies, we indicated progression in three stages for the neoplasms of each cell type (see below). Because hepatocytes and cholangiocytes are of epithelial origin, we used the term "carcinoma" to designate an obvious cancer.

"Hepatocellular alteration" designates focal, well-differentiated hepatocytic lesions, usually less than 1 mm in diameter, that are stained differently than the surrounding tissue (Figure 2a). These lesions are usually "basophilic", but may be "acidophilic", and occasionally the cells appear understained (i.e."clear cell"). These tinctorial changes reflect a combination of cytologic changes which may include decreased cytoplasmic glycogen, decreased iron deposits, and a variable increase or decrease in several enzymatic products. The net effect

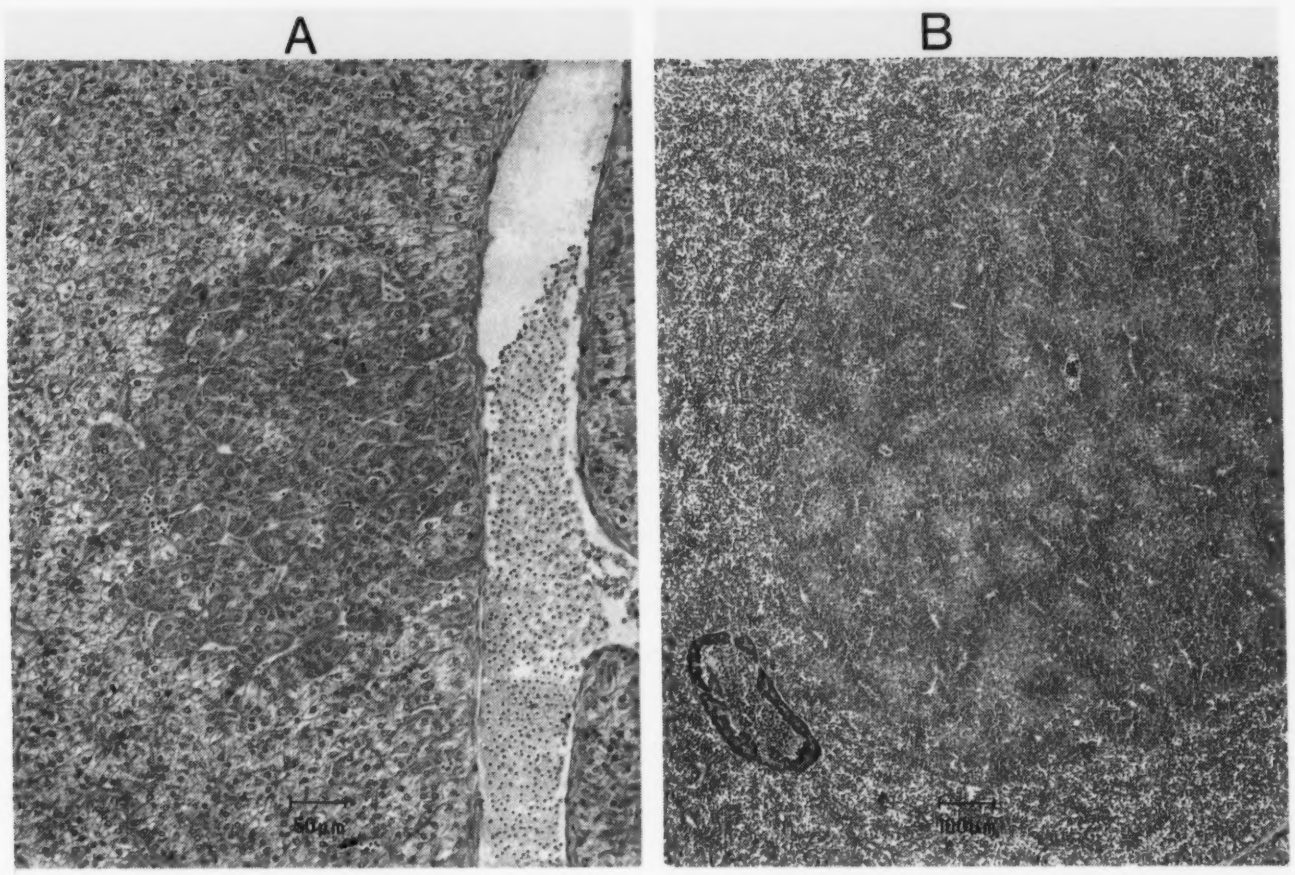

Figure 2: Hepatocytic lesions in a brown bullhead from the Black River: *(A) Hepatocellular alteration*: A basophilic focus borders on a large vessel. It has a normal pattern and cytology, except that cytoplasmic material is condensed due to reduced glycogen and other subcellular changes. The decreased cellular size is indicated by the surrounding narrow light band. 150x. *(B) Hepatocellular nodule*: An abnormal pattern and compression of adjacent normal liver occurs, in addition to the features in the hepatocellular alteration depicted in (A). 75x.

on cell size may be a slight increase, a slight decrease, or none. Mitotic figures are rarely apparent, indicating slow growth by the altered cells in the clone. The combination of slow growth with a possible slight change in cell size may result in a subtle compression of the surrounding tissue, or a subtle shrinking away from it. Usually, however, neither compression nor shrinkage is apparent, as the altered cords merge with those of the normal liver. Hepatocellular alteration certainly preceeds more advanced stages but it has not been established that hepatocellular alteration in brown bullheads always progresses. For this reason, these lesions were classified as pre-cancerous cellular alterations.

"Hepatocellular nodule" designates larger, more clearly defined subpopulations of hepatocytes than does "hepatocellular alteration". They are usually 1-4 mm in diameter and have a well differentiated trabecular pattern, with small alterations in cytology (Figure 2b). They compress or warp the pattern of adjacent normal tissue but do not exhibit overt signs of invasion. As in foci of hepatocellular alteration, hepatocellular nodules are usually basophilic but may be acidophilic or may consist of vacuolated "clear cells". Mitotic figures are scarce. Exocrine pancreas and macrophage aggregates are reduced or absent in the

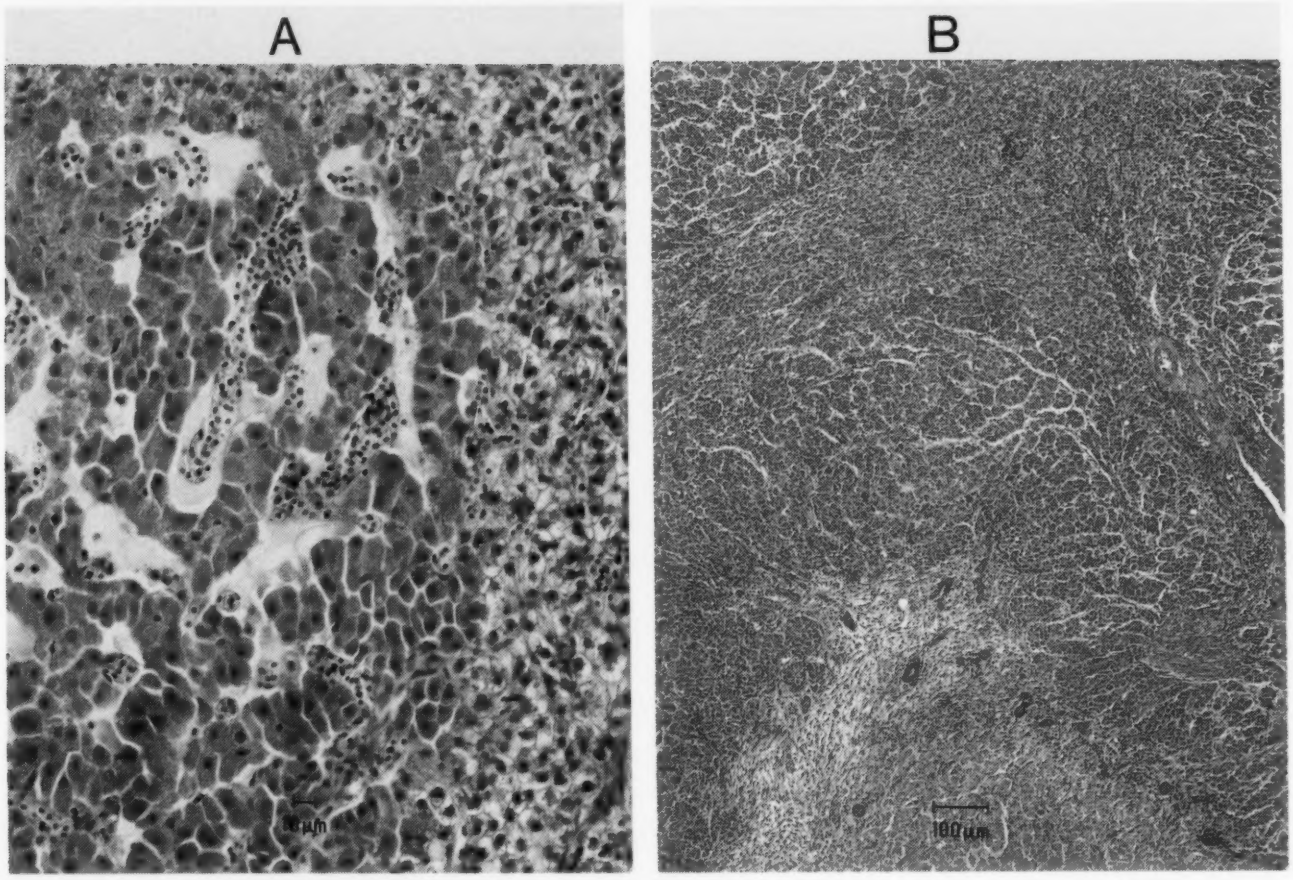

Figure 3: Hepatocarcinoma in brown bullheads. *(A)* Compared to the adjacent normal liver, the carcinoma exhibits cytomegaly and a trabecular pattern. 300x. *(B)* Portion of a large carcinoma with central necrosis (bottom, under scale) and compression of normal liver between two other cancers (top left and right). 75x. *(C)* Hepatocarcinoma metastasis in the spleen of a brown bullhead from a waterway other than the Black River. 200x.

nodules, compared to normal liver. Brown bullhead hepatocellular nodule is considered a benign intermediate stage, rather than the endpoint in the progression from a focus of hepatocellular alteration to frank hepatocellular carcinoma, for two reasons: a) they are part of a spectrum of lesions that bridge hepatocellular alterations and hepatocellular carcinomas, and b) Halver (1967) demonstrated by explant, implant and transplant studies that rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) hepatocellular nodules can progress to carcinomas. Hepatocellular nodules in brown bullhead resemble lesions sometimes called hepatocellular adenoma in rodents (Frith and Ward, 1980), but, in our view, the brown bullhead nodules have neither the glandular pattern nor the benign destiny implied by that terminology. Moreover, acceptance of the rodent terminology would also mean that the more advanced neoplasm, hepatocellular carcinoma, would have to be re-termed hepatocellular adenocarcinoma. However, we do accept "hepatoma" as a substitute for hepatocellular nodule. This lesion was classified as a non-cancerous neoplasms.

"Hepatocellular carcinoma" or "hepatocarcinomas" designates less well-differentiated masses, often with a solid or trabecular pattern, that are usually 0.4-1.5 cm in diameter (Figure 3a, Figure 3b). Tumor cells may be enlarged and pleomorphic. They are usually basophilic but may be acidophilic and rarely "clear cell". Mitotic figures are usually apparent and may be abundant. Invading fingers of tumor may interdigitate with normal

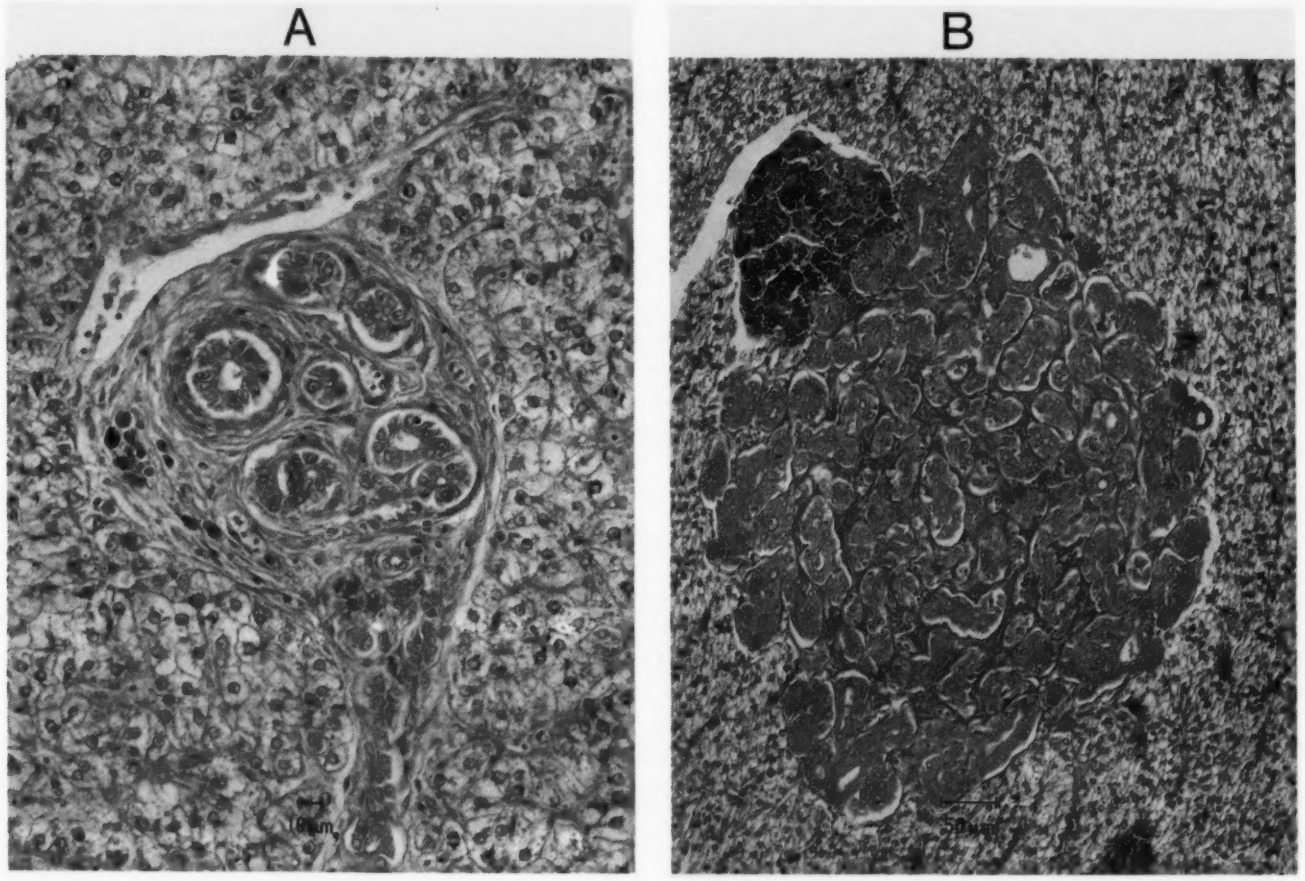

Figure 4: Biliary lesions in a brown bullhead from the Black River. *(A) Cholangiofibrosis*: A small cluster of bile ducts with periductular fibrosis has larger ducts centrally, and tiny sattelite ducts radiating out along the liver cords. 300x. *(B) Cholangioma*: A mass of well-differentiated cells appears to be enlarged by expansion rather than by aggressive invasion of the normal liver. 150x.

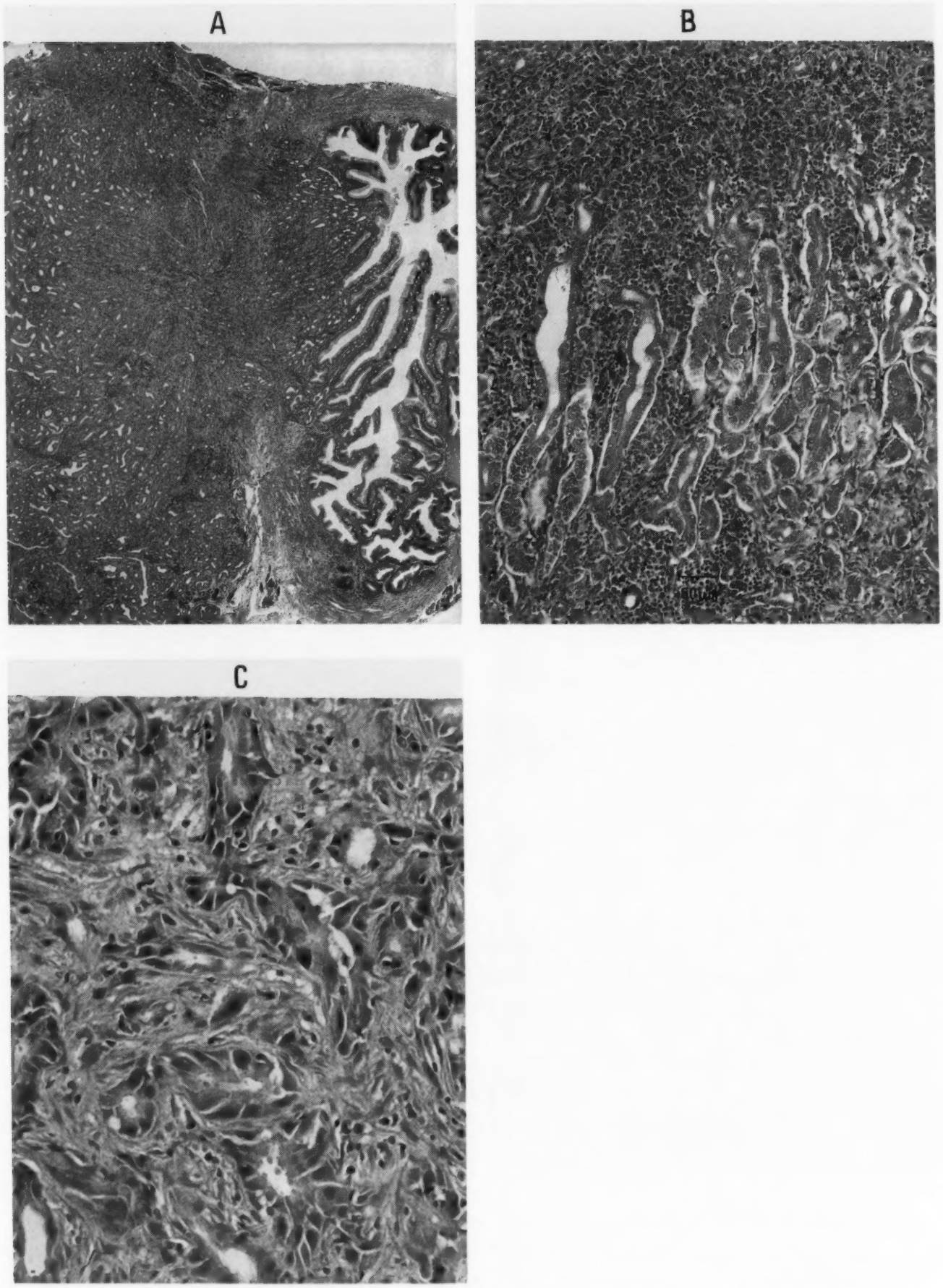

Figure 5: Cholangiocarcinoma in brown bullheads from the Black River. *(A)* A mass has invaded through the liver capsule into the adjacent gut, where it has destroyed the muscularis and penetrated to just under the mucosa. 30x. *(B)* The edge of a large mass has invading fingers interdigitating with normal tissue. 150x. *(C)* Poorly differentiated cancer cells are spindle-shaped and do not form ducts. 450x.

liver tissue at the interface. This lesion was classified as a cancer. Livers containing hepatocellular carcinoma often have multiple tumors per tissue section. Some may originate independently, others by extension, and still others by intraorgan metastasis. Interorgan metastases have been seen occasionally in spleen, kidney, and stannius corpuscles of brown bullhead (Figure 3c). Unfortunately, a systematic survey of other organs was not conducted on Black River bullheads to determine metastatic potential.

"Cholangiofibrosis" or adenofibrosis, as it is sometimes called in rodents (Firth and Ward, 1980), designates clusters of well-differentiated biliary ductules with increased periductular fibrosis (Figure 4a). Mitotic figures may be apparent, suggesting proliferation, and tiny satellite ductules may radiate out along liver cords, suggesting ductular metaplasia. Some of these lesions in brown bullheads probably represent toxic hyperplasia, and others may represent early neoplasia. This assumption, however, awaits experimental verification. This lesion was classified as a pre-cancerous cellular alteration.

"Cholangioma" designates well-differentiated ductular masses that have a relatively smooth, non-invasive interface with the normal liver (Figure 4b). Mitotic figures may be present. This lesion was classified as a non-cancerous neoplasm.

"Cholangiocarcinoma" designates poorly-differentiated, aggressive looking ductular lesions that range from less than 1 mm to more than 2 cm in size (Figure 5a-c). The amount of associated fibrosis can vary widely from almost none to scirrhus. The larger cholangiocarcinomas may be centrally necrotic. At the margins, long fingers of invading cancer often interdigitate with the normal liver. Mitotic figures may be numerous. This lesion was classified as a cancer.

Liver Tumor Frequencies

In 532 brown bullhead (>250 mm) collected from the Black River in 1981 and 1982, the average frequency of grossly visible liver tumors was 30.3% (Baumann et al., 1987). Bullheads of this length group from Old Woman Creek had no grossly observable liver tumors in either 1984 and 1985 (n=144). In a random sample of 10 bullhead livers from Old Woman Creek subjected to histopathological analysis, an area of hepatocellular alteration was found in one fish. The other 9 livers had no neoplastic or preneoplastic lesions.

In randomly sampled brown bullheads (>250 mm) from the Black River (n=125), the incidence of neoplasia diagnosed as cancer was 38.4% (Table 1). Only 20.8% had normal livers, with the others having either non-cancerous neoplasms or pre-cancerous cellular alterations. Although the frequency of advanced lesions diagnosed as cancers was 10% greater in age 4 fish than in age 3 fish, this difference was not statistically significant. Similarly, the frequency of advanced lesions in fish of ages 3 and 4 was about 10% higher in females than in males, but this difference was not significant.

The frequency of biliary cancer in Black River bullheads was higher in age 4 than in age 3 fish (Table 2), but the difference was not significant. However, when fish of ages 4 and 5 were combined (data not shown in Table 2), there was a significantly greater incidence of biliary cancer (35.5%) than fish of ages 2 and 3 combined (18.4%). The frequency of all biliary neoplasms (cholangiocarcinoma and/or cholangioma) was also significantly higher in fish of ages 4 and 5 than in those of ages 2 and 3 (46% and 26.5%, respectively).

Biliary cancer was significantly more prevalent than hepatocellular cancer in age 4 fish

Table 1.--Frequency of liver lesions in brown bullheads ≥ 250 mm long from the Black River, Ohio, in 1982.

Sample or subsample	N	% normal	% Cellular Alteration	% non-cancer neoplasm	% Cancer
All fish	125	20.8	19.2	21.6	38.4
Age 3 (both sexes)	48	22.9	20.8	25.0	31.2
Age 4 (both sexes)	73	19.2	19.2	20.5	41.1
Males (ages 3 and 4)	70	18.6	27.1	21.4	32.8
Females (ages 3 and 4)	51	23.5	9.8	23.5	43.1

TABLE 2

Frequency of hepatic and biliary lesions in subgroups of brown bullheads sampled from the Black River, Ohio, in 1982. Like notation indicates significant differences within columns (*,*f*) or rows (▲).

Lesion	Age 3 both sexes (n=48)	Age 4 both sexes (n=73)	Males Ages 3,4 (n=70)	Females Ages 3,4 (n=51)
a) Hepatic				
Hepatocellular alteration	16.7	16.4*	22.8	7.8
Hepatocellular nodule	18.8	13.7	17.1*	13.7
Hepatocarcinoma	16.7	16.4*f*	11.4*f*▲	25.5▲
b) Biliary				
Cholangiofibroma	10.4	6.8*	12.8	2.0
Cholangioma	8.3	11.0	5.7*▲	15.7▲
Cholangiocarcinoma	18.8	32.9*f*	27.1*f*	27.4

(sexes combined) and in males of ages 3 and 4 (Table 2). Moreover, the frequency of biliary cancer (30.8%) was significantly higher than that of hepatic cancer (10.2%) in age 4 males (n=39). However, hepatocellular alterations were observed significantly more frequently than cholangiofibroma in the age 4 fish that lacked more advanced neoplasms. Non-cancerous hepatocellular neoplasms (hepatocellular nodule) were generally more frequent than non-cancerous cholangiocellular neoplasms (cholangioma) in all subgroups. The prevalence of hepatocarcinomas and cholangiomas was significantly higher in females than in males. These trends held true within each of the individual age groups (3 and 4).

Age and Length Characteristics

The proportion of fish (>250 mm) that were age 5 or older was greater in Old Woman Creek than in the Black River (Figure 6). In fact, no brown bullheads older than age 5 were collected in the Black River in 1981 and 1982 (n=522), whereas brown bullheads of age 6 and 7 composed 18% of the 1984-1986 catch in Old Woman Creek. Similarly, brown bullheads of age 5 were almost six times more numerous in the Old Woman Creek collections than in the Black River collections. Fish of ages 3 and 4 together composed a

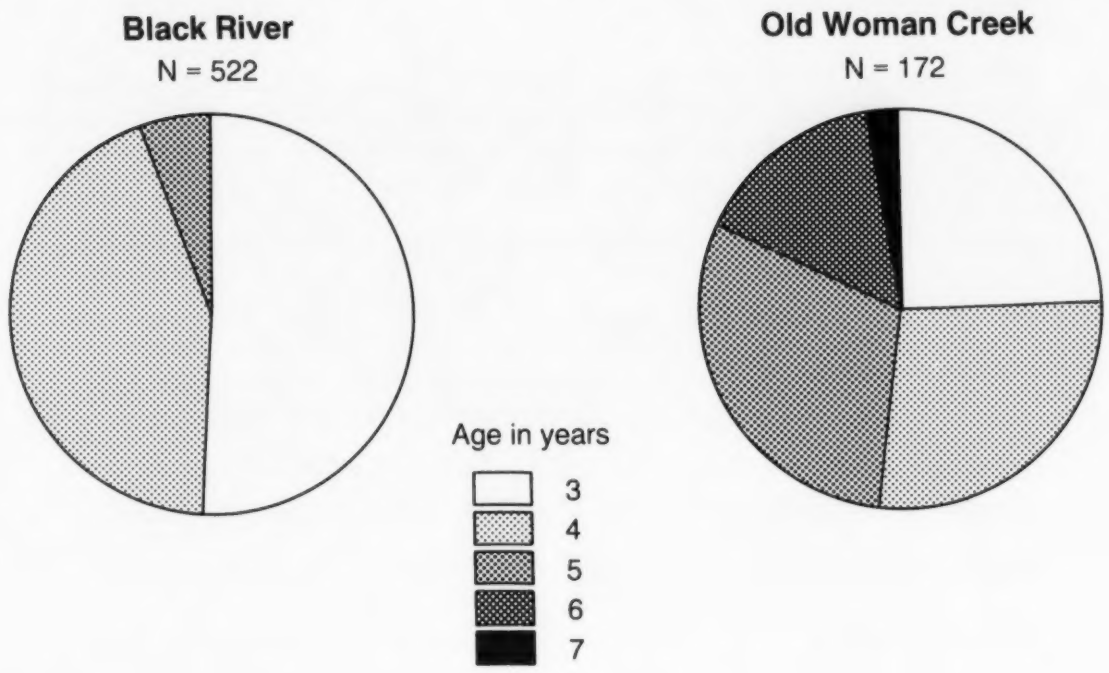

Figure 6: Proportion by age group of bullheads greater than 250 mm long collected in the Black River and Old Woman Creek.

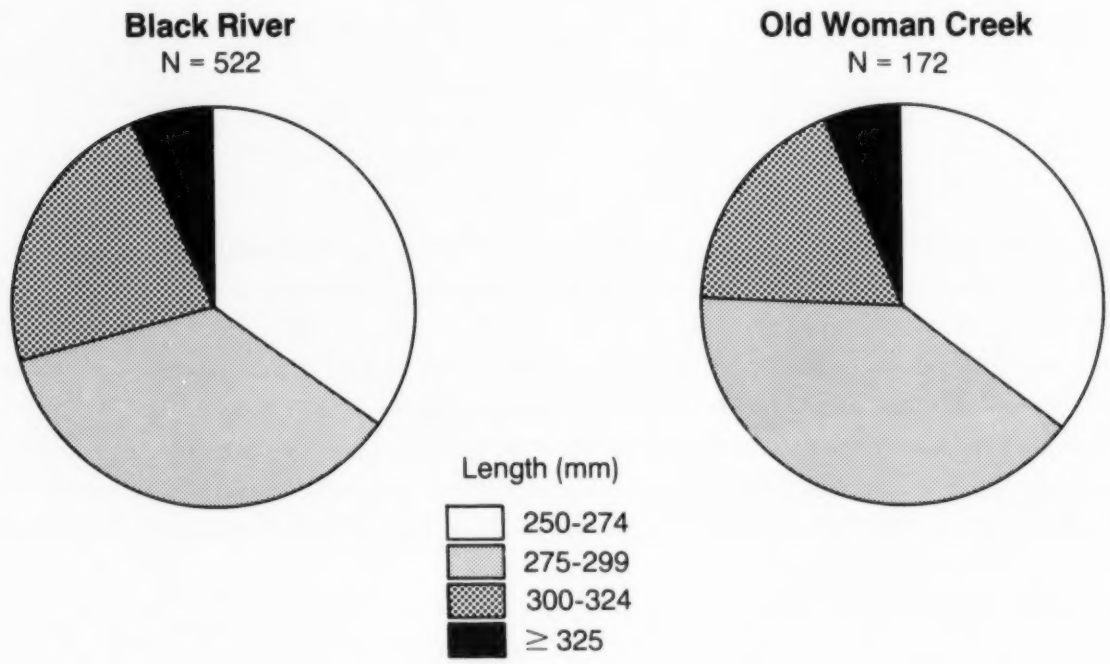

Figure 7: Proportion by length of bullheads greater than 250 mm long collected in the Black River and Old Woman Creek.

Table 3.--Length comparisons by age and season of brown bullheads taken from the Black River (BR) and Old Woman Creek (OWC), Ohio.

Season and location	Age	N	Length X	Length SD	t-value	p-value
Spring						
BR	3	164	282.2	21.5	-3.88	0.006
OWC	3	7	261.7	13.3		
BR	4	209	290.2	22.9	-2.55	0.019
OWC	4	17	278.6	17.7		
BR	5	27	307.2	29.8	-3.87	0.0004
OWC	5	27	280.5	19.8		
Fall						
BR	3	103	283.7	21.3	-1.80	0.075
OWC	3	35	277.1	17.7		
BR	4	18	287.6	24.9	-1.23	0.23
OWC	4	30	279.4	17.2		

significantly greater proportion of the catch in the Black River than in Old Woman Creek (Z test), and age 5 fish composed a significantly smaller proportion of the Black River catch. Spring collections in both systems resulted in higher proportions of older fish. From spring to fall, fish of age 5 and older declined from 6.8% to 0.8% of the catch in the Black River, and from 67.6% to 33.7% of the catch in Old Woman Creek. The Black River was sampled more heavily in spring (n=400) than in fall (n=122), whereas the sampling in Old Woman Creek was more evenly divided between spring (n=74) and fall (n=98). Thus, the catch in the Black River should have been weighted more heavily toward older fish than that in Old Woman Creek.

The length distributions of brown bullheads (>250 mm) from Old Woman Creek and the Black River were similar (Figure 7). In both waterways, from 6% to 7% of the fish taken were 355 mm long or longer, even though fish of age 5 constituted 5.4% of the Black River catch, and fish of ages 5-7 composed 48.2% of the catch in Old Woman Creek. Brown bullheads had a greater total length at a given age and season in the Black River than in Old Woman Creek, although these differences were statistically significant only for spring collections (Table 3). Fall collections of age 5 bullheads could not be compared, because only a single fish of age 5 was caught in the Black River. In fish from both streams, increased length corresponded to increased age.

DISCUSSION

A high frequency of grossly observable liver tumors was reported for brown bullheads collected from the Black River in 1980-1982 (Baumann et al., 1982, 1987). The absence of grossly-visible liver tumors in bullheads from a reference site, Old Woman Creek, corresponds to data from other reference locations, such as Buckeye Lake, Ohio (Baumann et al., 1987), and Lake of the Woods, Ontario (Brown et al., 1973).

The hepatocellular and cholangiocellular neoplasms observed in Black River bullheads have also been observed in brown bullheads from the Buffalo River (J. Black, personal communication). Several other populations of feral fish have been reported to have both biliary and hepatic carcinomas, including white suckers (*Catostomus commersoni*) from sites in Lake Ontario (Hayes et al., this volume), English sole (*Parophrys vetulus*) from various locations in Puget Sound (Malins et al., 1984), and winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor, Massachusetts (Murchelano and Wolke, 1985).

The frequency of grossly visible liver tumors increased with age in Black River bullheads (Baumann et al., 1987), and all tumors that were grossly visible were diagnosed as cholangiocellular carcinomas (Baumann and Harshbarger, 1985). The frequency of biliary cancers was significantly higher in the older (age 4 and 5) brown bullheads diagnosed in this study. A positive correlation between fish age and tumor frequency is compatible with the hypothesis of a chemical etiology, in which a latent period between initiation and tumor development would be expected. Young English sole (under 4 years of age) seldom had liver neoplasia (Malins et al., 1980), even though covalent binding of benzo(a)pyrene (BaP) metabolites to liver DNA was 10 times greater in sole of age 3 than in fish of age 6 and older fed BaP (Varanasi et al., 1985). Polynuclear aromatic hydrocarbons (PAHs) and their metabolites, including BaP, have been implicated in the etiology of liver neoplasms in Black River bullheads (Baumann and Harshbarger, 1985; Baumann et al., 1987).

In contrast to cholangiolar carcinomas, the frequency of hepatocarcinomas in Black River bullheads remained relatively constant between ages 3 and 4. These data suggest that hepatocellular and biliary lesions may have different mechanisms of initiation or promotion, or may grow at different rates. The significantly higher incidence of cholangiocellular than hepatocellular cancers in age 4 bullheads from the Black River is similar to relative frequencies of 7% cholangiocellular and 2.5% hepatocellular lesions reported for the two cancers in winter flounders (Murchelano and Wolke, 1985), but differs from the cancer ratios reported by Malins et al. (1985a) for English sole near Mukilteo (1.5% cholangiocellular and 4.6% hepatocellular), and by Malins et al. (1985b) for English sole in Eagle Harbor (8% cholangiocellular and 20% hepatocellular). Both winter flounder and English sole were reported to have a higher frequency of cellular alterations than of carcinomas. Direct comparisons of neoplasm frequencies are difficult, since most tumor prevalence data previously reported have not been analyzed by age or sex.

Differing frequencies of hepatocarcinoma for males and females have not previously been reported for a feral fish population. A recent study by Jodon (1987) found no difference by sex in the aryl hydrocarbon hydroxylase activity of mature Black River bullheads, and Varanasi et al. (1982) showed that male and female English sole exposed to BaP had similar levels of DNA binding and proportions of conjugates in bile. However, Fabacher and Baumann (1985) reported that amounts of hepatic microsomal protein (mg g^{-1}) in tissue and cytochrome b_5 (nmol g^{-1}) in liver tissue were both greater in Black River females than males, for bullheads with no tumors. This relationships did not hold for fish with tumors. No hypothesis is readily available to explain why the incidence of hepatocellular cancer, but not of biliary cancer, was significantly higher in females than in males in our study.

In Muskellung Lake, New York, age 5 fish constituted 26.6% of the brown bullheads of ages 3 and older, and fish of ages 6 and 7 made up 19% of the population (n=998; Sinnot and Ringler, 1987). This age distribution in the Muskellung Lake population was similar to that in the Old Woman Creek population. The Black River population, however, lacked the two oldest age groups and included only 5.4% of age 5 fish. Recruitment could not be estimated at the two sites. Slower recruitment of age 3 and 4 fish in Old Woman Creek could increase the proportion of older age groups captured, but the absence of age 6 and 7 fish in the Black River verifies that there is a real difference in age composition between the two sites.

A variety of factors, other than liver tumors, may be responsible for the altered age composition in the Black River. Older age classes in fish populations are often eliminated by mortality caused by angling (Goedde and Coble, 1981; Frie and Spangler, 1985). However, because access to the industrialized lower reach of the Black River is severely limited, angling pressure appeared to be light. Furthermore, angling pressure, which is size-selective rather than age-selective, usually eliminates larger length classes and results in a population with a smaller median size (Goedde and Coble, 1981; Frie and Spangler, 1985). This was not the case in the Black River. Liver parasitism was less prevalent in the Black River than at other Great Lakes locations (Baumann, unpublished data), and the rapid growth rate of the Black River fish indicates that food availability was adequate at this site.

The absence of older age classes might also be caused by differential emigration of older fish out of the river. However, evidence for such differential emigrations in bullheads is lacking, and an explanation for why mature fish of 3 and 4 years remain in the river while

5, 6, and 7 year-olds emigrate would be difficult to postulate. Therefore, the age distribution data for bullhead from the Black River is consistent with an age-selective mortality caused by liver carcinomas or the stress associated with liver carcinomas. The hypothesis which best fits the available data is that brown bullheads in the Black River were subjected to a direct or indirect mortality from hepatic cancer that increased with age, and that resulted in the elimination of fish older than age 5 from the population.

ACKNOWLEDGEMENTS

We appreciate the field assistance of William L. Parland, Eric P. Johnston, Tracy A. Jodon, and Vanessa M. Leiby. We thank David L. Fabacher and Christopher J. Schmitt for valuable reviews of the manuscript, Kathy Price and Jennifer Clark for the histotechnology, and Nancy Demarchi for manuscript preparation. This research was supported in part by the National Institute of Health contract NO1 CP 61063.

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FOOTNOTES

[1] Reference to trade names does not imply endorsement by the U.S. Fish and Wildife Service.

The Science of the Total Environment, 94 (1990) 89–104
Elsevier

32P-POSTLABELING DETECTION OF DNA ADDUCTS IN FISH FROM CHEMICALLY CONTAMINATED WATERWAYS

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ABSTRACT

Fish were collected from sites in the chemically-contaminated Buffalo River, New York, and the Detroit River, Michigan. The sediments of these rivers have high levels of chemical contaminants, including polycyclic aromatic hydrocarbons (PAHs), and fish from these locations have high prevalences of liver cancer. To determine chemical-DNA interactions and a possible role for chemicals as a cause of the observed tumors, DNA was isolated from livers and was enzymatically digested to normal and adducted nucleotides. The DNA digests were enriched for hydrophobic, bulky adducts, either by preparative reverse phase high pressure liquid chromatography, or by selective nuclease P1 dephosphorylation of normal nucleotides. DNA-chemical adducts were then quantitated by ^{32}P-postlabeling analysis. Regardless of the adduct enrichment procedure, the chromatograms derived from DNA of fish from polluted areas showed a diffuse, diagonal radioactive zone consisting, at least in part, of multiple overlapping discrete adduct spots. The behavior of the adducts in the diagonal radioactive zone and of their unlabeled precursors is consistent with their identification as nucleotide adducts of a variety of bulky, hydrophobic, aromatic genotoxic compounds. Analysis of bile demonstrated recent exposure to multi-ringed aromatic compounds.

INTRODUCTION

An increasing number of epizootics of tumors in wild fish populations have been reported during the past decade (Couch and Harshbarger, 1985). Many areas that have been studied have a history of chemical pollution from industrial, municipal, and agricultural sources and have sediments that are contaminated with polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons, and heavy metals. It has been suggested that such sediment-bound chemical contaminants may be involved in causing the observed neoplasia (Black, 1984; Couch and Harshbarger, 1985). The relationship between sediment-bound PAHs and tumors in wild fish has been of particular interest because several known carcinogens such as benzo(a)pyrene (BaP) and benzanthracene are found in this class of

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chemicals. In at least three areas, the Buffalo River, New York, the Black River, Ohio, and Puget Sound, Washington, high levels of aromatic hydrocarbons found in the sediments have been linked to tumors in bottom-dwelling fish (Baumann et al., 1982; Black, 1983a; Black et al., 1985a; Malins et al., 1984; Malins et al., 1985). Laboratory studies have demonstrated that fish have hepatic enzymes capable of metabolizing BaP to reactive intermediates that bind to DNA (Ahokas et al., 1979; Von Hofe et al., 1980; Varanasi et al. 1981; Varanasi et al., 1986; Hinton et al., 1981; Nishimoto and Varanasi, 1985). Moreover, tumors have been induced in fish exposed to BaP or other PAHs in laboratory experiments (Ermer, 1970; Schultz and Schultz, 1982; Hendricks et al., 1985; Black et al., 1985b; Hawkins et al., 1988). These studies are suggestive of a causal relationship between exposure to sediment-bound aromatic hydrocarbons and neoplasia in wild fish species.

It is generally believed that the initiating event for chemical carcinogenesis is covalent binding of chemicals or their metabolites to DNA, resulting in the formation of carcinogen-DNA adducts (Ruddon, 1987). Detection of such DNA adducts in fish tissues would be a direct measure of genetic damage resulting from exposure to environmental carcinogens. Traditionally, radiolabeled chemicals have been used to study DNA adduct formation (Martin and Garner, 1987). However, given the large number of potential environmental genotoxic agents of unknown structure and concentration that might be found in a chemically contaminated waterway, a more general method for detecting DNA adducts is needed. The recently developed ^{32}P-postlabeling techniques provide such a method (Randerath et al., 1981; Gupta et al., 1982). In this study, we used this sensitive technique, which can detect one DNA adduct in 10^9 bases, to analyze liver tissues from fish collected from chemically contaminated waterways for the presence of chemical-DNA adducts.

MATERIALS AND METHODS

Reagents and Chromatography Materials

Preparation and sources of enzymes and other reagents for ^{32}P-postlabeling and related methods have been previously described (Dunn et al., 1987). High specific activity [γ^{32}P] -ATP (>3000 Ci mmole^{-1}) was either synthesized according to Gupta et al. (1982) or purchased from ICN, Irvine, California. High pressure liquid chromatography (HPLC) columns for the chromatography of both nucleosides and 3'-nucleotides were custom-packed by Alltech Associates, Deerfield, Illinois. Columns were 2.1 mm inside diameter x 100 mm and contained 5 μm ODS-3 reverse-phase packing manufactured by Whatman, Clifton, New Jersey. PEI cellulose thin layer chromatography plates (Machery Nagel, West Germany) were purchased from Brinkmann Instruments, Rexdale, Ontario, or Alltech Associates Deerfield, Illinois.

Fish

Brown bullheads (*Ictalurus nebulosus*), walleye (*Stizostedion vitreum*), white suckers (*Catostomas commersoni*), and common carp (*Cyprinus carpio*) were collected from two polluted Great Lakes tributaries by electroshocking. Fish from the Buffalo River, New York were obtained from an area approximately 1 km from the mouth of the river. Fish

from the Detroit River, Michigan were obtained from sediment deposition zones within the Trenton Channel. After collection, fish were placed on ice and returned to the laboratory for processing. Laboratory-raised brown bullheads were collected by nets from a pond free from industrial pollution and were raised in aquaria for 9 mo prior to sacrifice. During this time, fish were fed commercially available pelleted trout food. Fish were killed by a blow to the head and examined for external signs of pathology. The liver and gall bladder were removed and separated, and pieces of liver were fixed in 10% neutral buffered formalin for histological preparation. The remainder of the liver was frozen in liquid nitrogen or acetone and dry ice and stored at -70°C until processed for DNA extraction. Bile, when present, was removed from the gall bladder, frozen in dry ice, and stored at -20°C until analyzed.

Histological Processing of Liver Tissue

Tissues that had been fixed in 10% neutral buffered formalin were dehydrated through a graded series of alcohol. Dehydrated tissues were then embedded in paraffin blocks and sections of 6 μm thickness were made. Sections were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin (H&E). H&E stained material was examined by light microscopy.

Analysis of Bile Samples

Bile was analyzed for metabolites of multi-ringed aromatic compounds as previously described (Krahn et al., 1984; Maccubbin et al., 1988). Samples were injected without pretreatment onto a Perkin Elmer HPLC equipped with a C_{18} reverse-phase column (Vydac 201TP55, Separations Group, Vesperia, California). The column was eluted with a linear gradient of 100% water to 100% methanol in 15 min, followed by 25 min at 100% methanol. Eluted materials were detected by fluorescence spectrometry at 380 nm excitation and 430 nm emission. Fluorescing compounds were quantitated in terms of BaP equivalents and expressed in ng BaP equivalent per g bile, as previously described (Krahn et al., 1986).

Processing of Liver for 32P-Postlabeling

The procedures for processing liver tissue and subsequent ^{32}P-postlabeling are outlined in Figure 1. These methods have been described in detail elsewhere (Gupta et al., 1982; Reddy et al., 1984; Reddy and Randerath, 1986; Dunn et al., 1987). Briefly, DNA was isolated from tissue by enzymatic digestion, solvent extraction, and ethanol precipitation (Maniatis et al., 1982; Dunn et al., 1987). An aliquot (2.5 - 5.0 μg) of isolated DNA was digested enzymatically to deoxynucleoside-3'-monophosphates (dN3'P) by treatment with micrococcal nuclease and spleen phosphodiesterase. The digest was then processed to remove normal dN3'Ps, thus enriching the digest for DNA adducts. Enrichment was by either HPLC separation (Dunn et al., 1987) or nuclease P1 treatment (Reddy and Randerath, 1986). Several digested DNA samples from brown bullheads were enriched by both procedures in order to compare the techniques. Chemical-DNA adducts in the digest were labeled by a kinase reaction, transferring ^{32}P from [γ ^{32}P]ATP to the 5' end of the

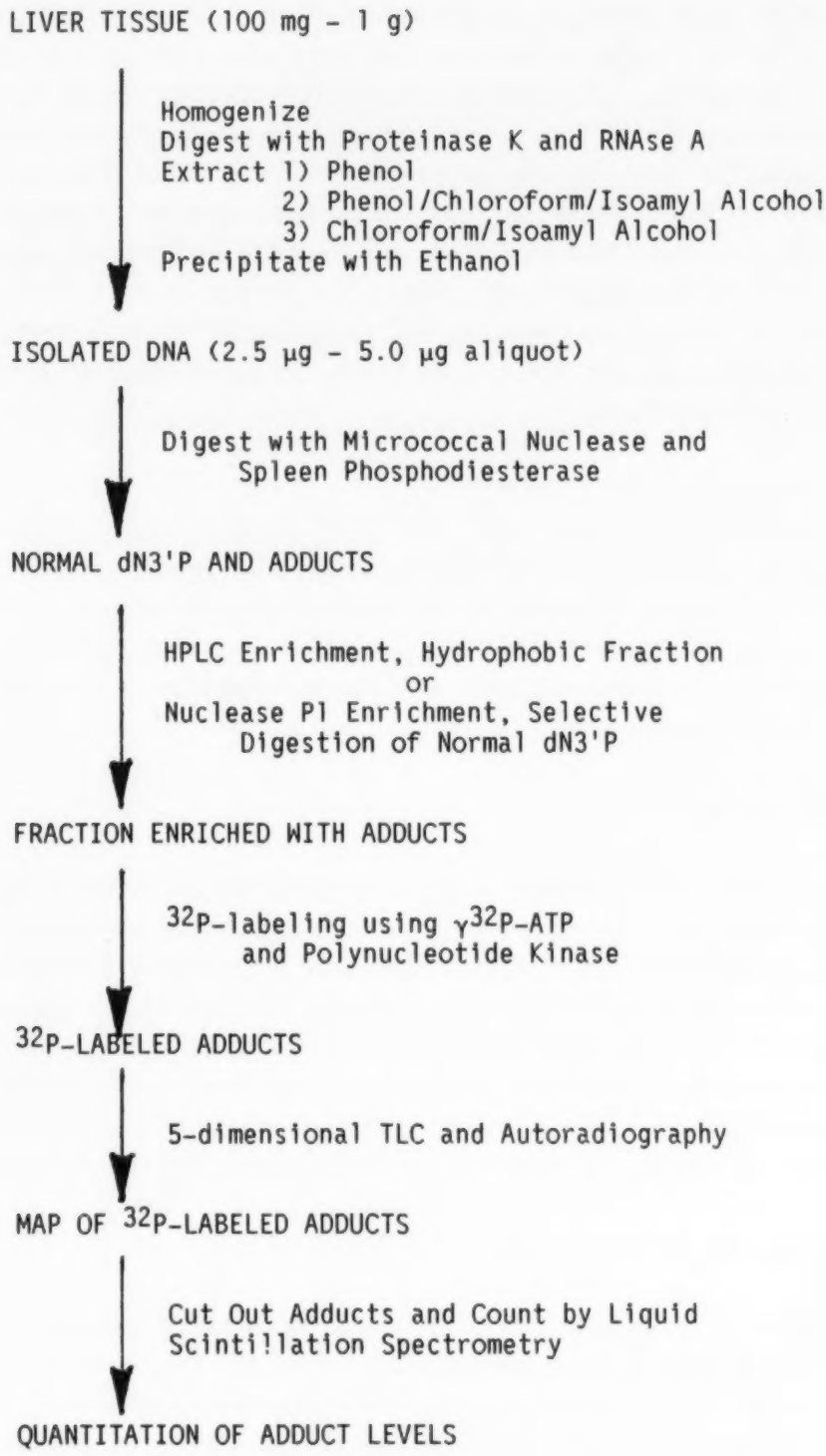


Figure 1: Processing of liver tissue for ^{32}P-postlabeling.

nucleotide using polynucleotide kinase. After ^{32}P-postlabeling, samples were spotted on PEI cellulose TLC plates and developed by 5 dimensional chromatography as previously described (Gupta et al., 1982; Dunn and Stich, 1986).

Quantitation of Adduct Levels

TLC plates were placed in cassettes with X-ray film and two intensifying screens. Adducts were detected by autoradiography for 16-24 h at room temperature or, for increased sensitivity, at -70°C. The specific activity of the [γ^{32}P]ATP used in each kinase reaction was determined by chromatography of postlabeled deoxyadenosine-3'-monophosphate (four 5 μl samples of 10^{-7} M solution) on PEI cellulose with 0.3 M ammonium sulfate buffered with 10 mM sodium phosphate to pH 7.5 (Dunn and Stich, 1986). Spots corresponding to [^{32}P]deoxyadenosine-3',5'-biphosphate were cut out and their radioactivities were determined by liquid scintillation counting. The specific activity of the [γ^{32}P]-ATP was calculated by assuming 100% labeling of the defined amount of nucleotide. The specific activity typically was >3000 Ci mmol^{-1}.

Sections of chromatograms containing adducts were cut out and their areas were estimated by weighing the cut pieces of chromatogram. The level of radioactivity in these sections was determined by liquid scintillation counting using a toluene-based scintillation fluid, giving a counting efficiency of essentially 100%. Background levels of radioactivity in the cut-outs were estimated by determining the radioactivity in several 1 cm^2 areas adjacent to the zone. This background, typically between 100 and 200 dpm per cm^2, was subtracted from the radioactivity in the adduct zone, which, in these experiments, typically covered an area of 15 cm^2. After background subtraction, the moles of adducts in the chromatographic zones were calculated by using the net amount of radioactivity and the known specific activity of the [γ^{32}P]ATP used in the ^{32}P-postlabeling reaction.

Finally, the concentration of adducts (nmol mol^{-1}) in the DNA digests was calculated by dividing the moles of adducts by the total moles of nucleotides as determined by HPLC. For HPLC enriched samples, the total moles of normal DNA nucleotides were determined by HPLC quantitation of deoxyadenosine-3'-monophosphate observed during the isolation of hydrophobic adducts. Similar procedures were used for nuclease P1 enhancement samples, except quantitation of moles of normal nucleotides were based on HPLC analysis of the amount of deoxyguanosine in a measured aliquot of the nuclease P1 digest.

RESULTS

Histopathology

Only brown bullheads from the Buffalo River and walleye from the Detroit River had liver neoplasms (Table 1). Liver pathology in the form of foci of altered hepatocytes was observed in 26.9% (7 of 26) of the bullheads collected from the Buffalo River, and two of these fish also had bile duct neoplasms. Typical examples of these lesions are illustrated in Figure 2. One walleye of the five collected from the Detroit River had a hepatocellular neoplasm.

Bile Analysis

Analysis of bile by HPLC indicated that most of the wild fish had been recently exposed to multi-ringed aromatic compounds. Chromatograms of bile samples (Figure 3) were usually characterized by a complex array of peaks fluorescing at wavelengths selective for

Table 1. Summary of adduct levels in liver, liver tumor incidence, and BaP equivalents in bile in laboratory raised and wild fish.

Species[a]	Source[b]	Adduct Levels[c]	BaP Equivalents[d]	Tumor Incidence[e]
BH	BR	90 ± 43 (N)	3372 ± 2128	7/26
		70 ± 42 (H)		
	DR	179 ± 42 (N)	828 ± 688	0/6
		54 ± 28 (H)		
	LAB	18 ± 13 (N)	248 ± 159	0/6
		15 ± 9 (H)		
WS	BR	439 ± 127 (N)	ND	0/3
C	BR	429 ± 189 (N)	22,883 ± 18,409	0/3
W	DR	248 ± 100 (N)	3246	1/5

a) BH = brown bullhead; WS = white sucker; C = carp; W = walleye.

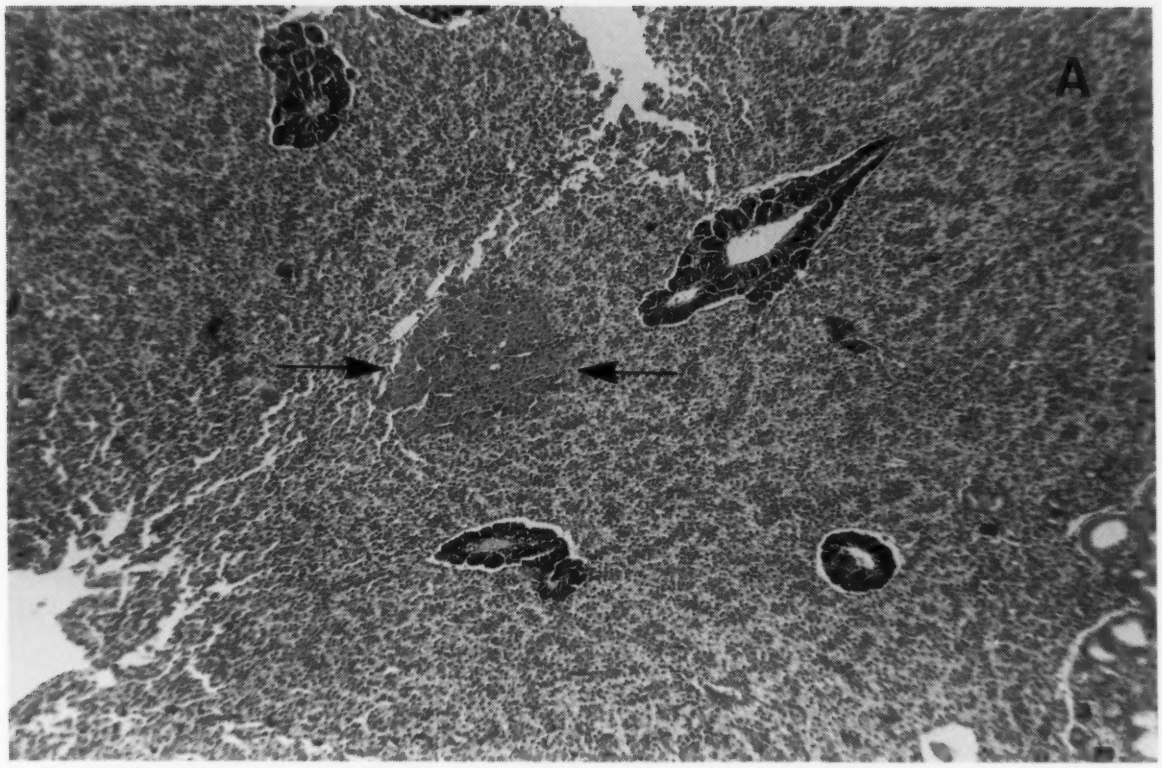
b) BR = Buffalo River; DR = Detroit River; LAB = laboratory raised.

c) Adduct levels are in nmole adduct/mole normal nucleotide. Values are mean ± standard deviation. (N) = Nuclease P1 enrichment; (H) = HPLC enrichment.

d) Values are in ng BaP equivalents/g of bile, wet weight. Values are mean ± standard deviation except walleye in which only a single fish had bile. ND = not determined.

e) Values are # of fish with lesion/# of fish examined. Included as tumors are foci of altered hepatocytes, bile duct and hepatocellular neoplasms.

multi-ringed aromatic compounds (Krahn et al., 1987). These compounds eluted from the HPLC column earlier than BaP, and thus were presumed to be polar metabolites of multi-ringed aromatic compounds. When quantitated in terms of BaP equivalents, levels of fluorescent compounds were higher in the bile of fish from the Buffalo River than levels in fish from the Detroit River (Table 1). For example, brown bullheads from the Buffalo River had a median value of 3,372 ng BaP equivalents in bile compared to a median value of 828 ng BaP equivalents in the bile of Detroit River bullheads.



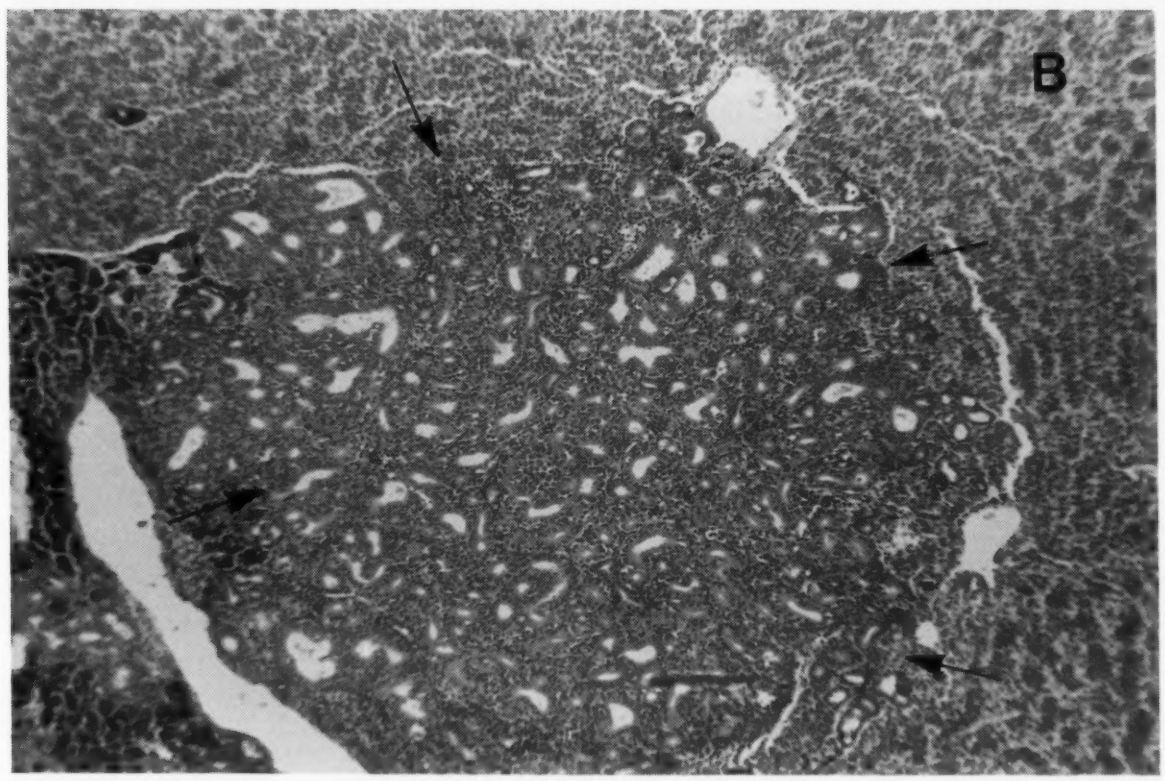


Figure 2: Hepatic lesions observed in brown bullheads collected from the Buffalo River: a) focus of altered hepatocytes, eosinophilic type, b) bile duct neoplasm.

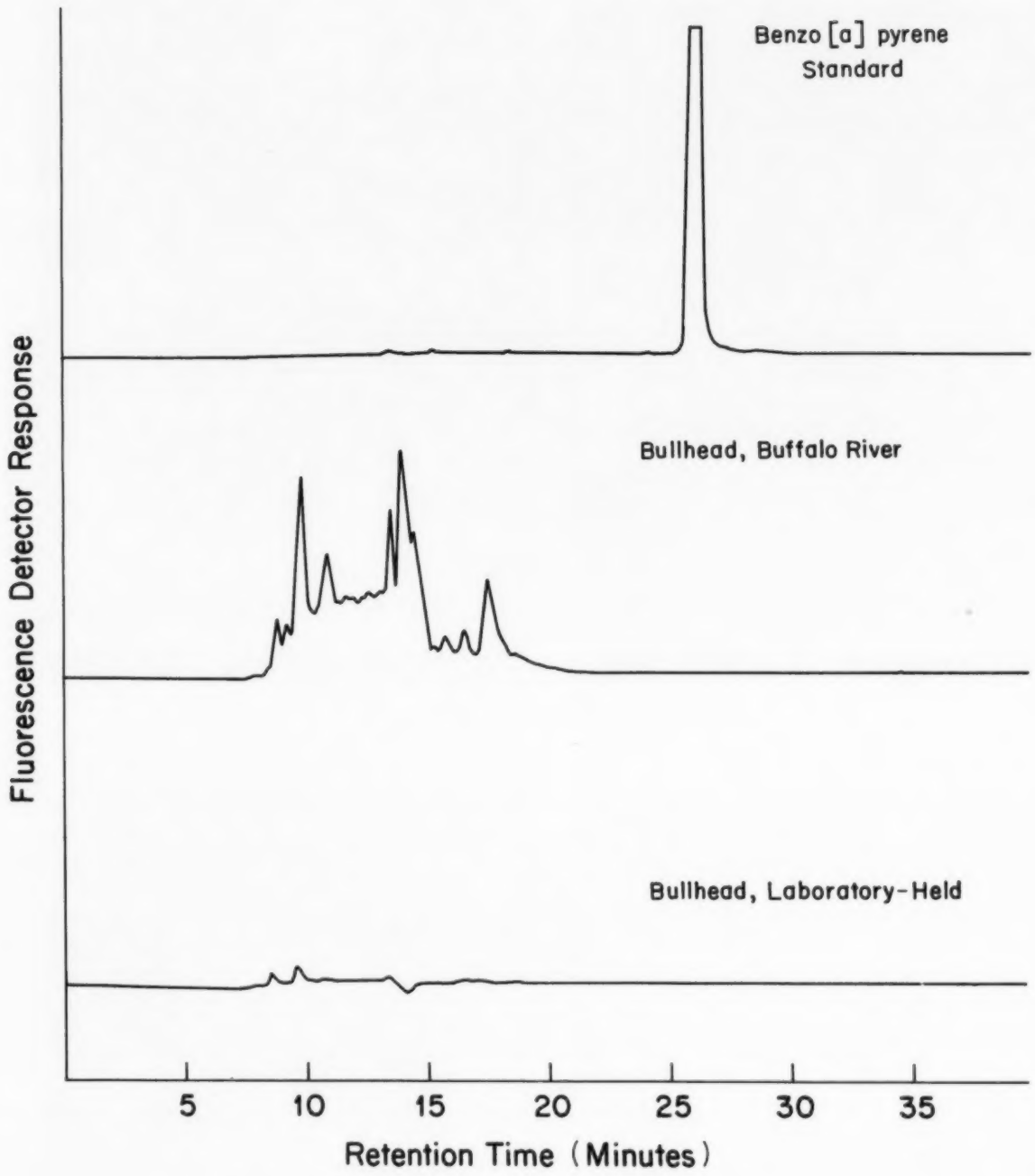


Figure 3: HPLC chromatograms of bile taken from brown bullheads, detected by fluorescence at wavelengths 380nm/430nm (excitation/ emission).

32P-Postlabeling of DNA Adducts

Figure 4 shows representative autoradiograms of chromatograms derived from DNA isolated from the livers of the four fish species collected from the Buffalo and Detroit Rivers. All of the autoradiograms in this figure were derived from TLC maps of DNA treated by nuclease P1 enrichment of adducts prior to ^{32}P-postlabeling. Each chromatogram exhibits a diffuse diagonal zone of radioactivity with a varying number of more intense

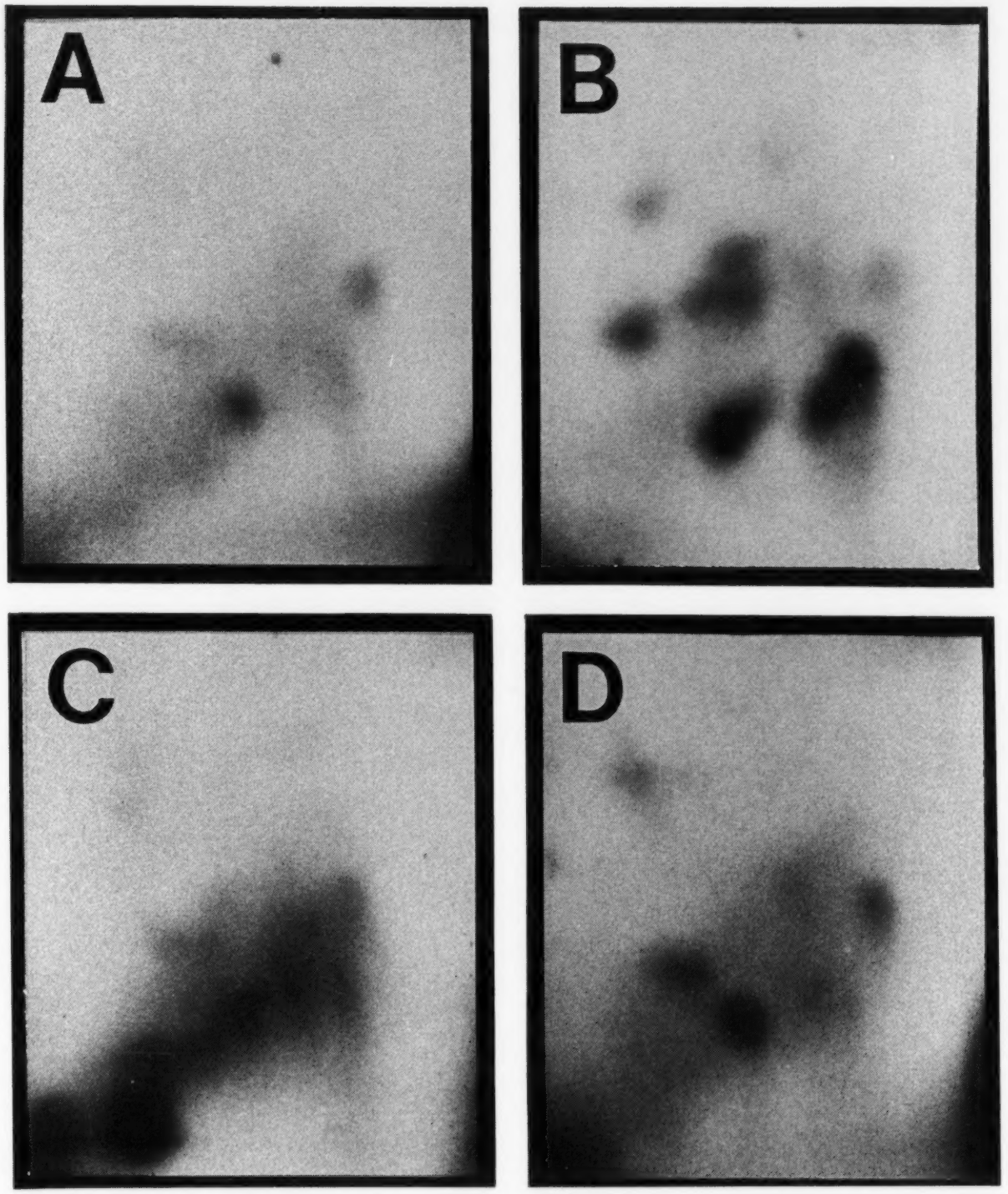


Figure 4: Autoradiograms of chromatograms showing chemical-DNA adducts in livers of wild fish after nuclease P1 enrichment and ^{32}P-postlabeling: a) brown bullhead, Detroit River; b) walleye, Detroit River; c) white sucker, Buffalo River; d) carp, Buffalo River.

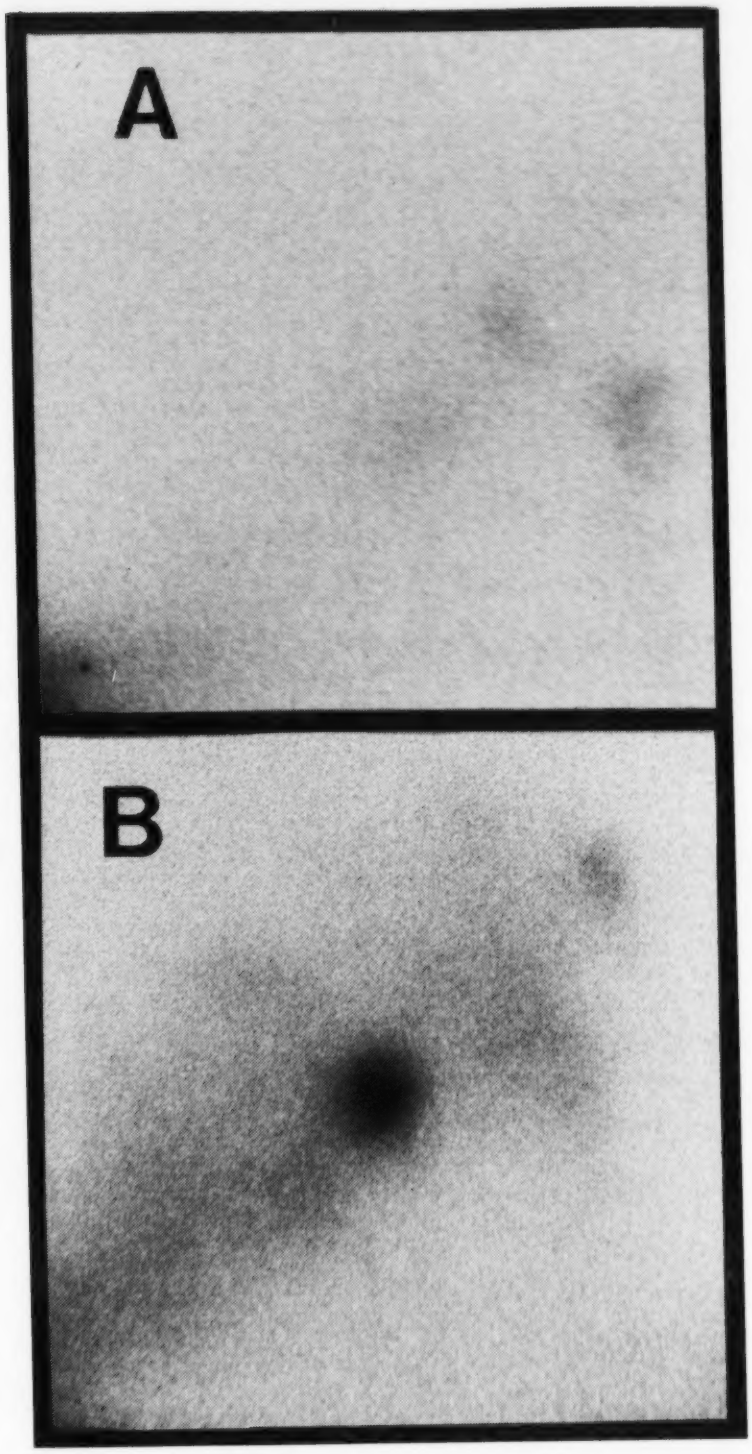


Figure 5: Comparison of chemical-DNA adduct levels in the liver of bullheads taken from a laboratory-raised group (a), and from the Detroit River (b). DNA analyzed by nuclease P1 enrichment.

spots visible within the zone. Chromatograms of HPLC-enriched samples had patterns of adducts similar to chromatograms from nuclease P1-enriched samples. The pattern of adducts, though similar for all fish, showed variation in intensity of spots within the diagonal zone from one individual to another. No consistent pattern was observed with respect to species or site of collection. In contrast, chromatograms of DNA from laboratory-raised bullheads had a faintly visible diagonal radioactive zone with faint discrete spots when analyzed under the same conditions as bullheads collected from the Buffalo and Detroit Rivers (Figure 5).

Adduct levels in individual fish collected from the Buffalo and Detroit Rivers and analyzed using nuclease P1 enrichment are shown in Figure 6. The adduct levels in individuals ranged from 32 nmol mol^{-1} to 642 nmol mol^{-1}. Average adduct levels analyzed after nuclease P1 or HPLC enrichment for the limited sample sizes are summarized in Table 1. Adduct levels after nuclease P1 enrichment in brown bullheads from the Detroit and Buffalo Rivers were compared to those found in laboratory-raised brown bullheads using the Mann-Whitney test. Brown bullheads from the contaminated waterways had significantly higher adduct levels ($P<0.01$). At the present time, no statistical analyses of other species is possible because of the lack of fish from uncontaminated reference areas.

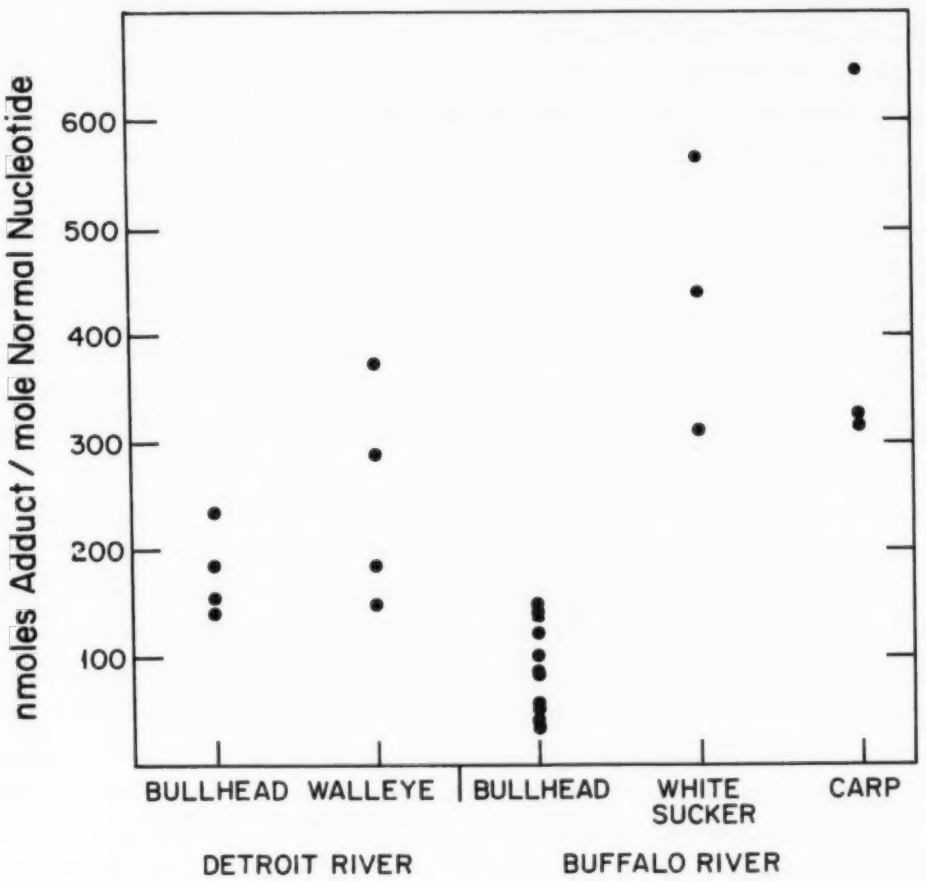


Figure 6: Chemical-DNA adduct levels in liver of individual fish from the Buffalo River and Detroit River. DNA analyzed by nuclease P1 enrichment.

However, in the limited sample of walleye, carp, and white suckers, adduct levels were on average greater than 150 nmol mol^{-1}.

DISCUSSION

In this study, we have demonstrated the potential for using ^{32}P-postlabeling to detect chemical-DNA adducts in fish tissues. This method allows detection of the adducts without prior knowledge of the chemicals to which the fish have been exposed. Brown bullheads collected from the Buffalo and Detroit Rivers had increased levels of DNA adducts, relative to laboratory-raised fish, that were visible as a diffuse radioactive zone with various numbers of intense spots. At present, the exact identity of these adducts is unknown. However, based on their behavior in our HPLC system and in the TLC system and their resistance to nuclease P1 digestion, we can make some generalizations about their structure.

Compounds found in the radioactive zone after HPLC treatment are hydrophobic in nature. They were retained on a reverse-phase column under conditions in which normal nucleotides elute rapidly. We have previously reported (Dunn et al., 1987) that many of the compounds in the radioactive zone are more hydrophobic than the major DNA adduct formed by BaP. The solvent systems used for development of TLC plates selectively removes nonaromatic and hydrophilic materials from the TLC plates in the first two dimensions and then separates materials containing an aromatic or bulky lipophilic moiety (Gupta et al., 1982; Reddy and Randerath, 1986). The chromatograms of fish DNA indicated that there were a wide variety of aromatic, lipophilic compounds bound to the nucleotides. Nuclease P1 selectively dephosphorylates normal dN3'Ps to nucleosides which are not substrates for polynucleotide kinase. Nucleosides which have bulky adducts are resistant to the 3'-dephosphorylase activity of nuclease P1 and thus remain as substrates for the kinase (Reddy and Randerath, 1986). Our results demonstrate the presence of resistant DNA adducts and therefore suggest that bulky chemicals are adducted to the fish DNA. Both the Detroit and Buffalo Rivers are known to have sediments contaminated with PAHs (Black, 1983b; Fallon and Horvath, 1985). Given the general characteristics of the DNA adducts observed by ^{32}P-postlabeling, it is possible that at least some of these adducts resulted from exposure to sediment-bound PAHs.

Analysis of bile indicated that the fish collected from the Detroit and Buffalo Rivers had been recently exposed to multi-ringed aromatic compounds such as pyrene, fluoranthene and BaP. Materials observed in the bile were detected by fluorescence at wavelengths selective for this class of compounds (Krahn et al., 1987), and were more polar than unmetabolized BaP. Concentrations of PAH compounds in sediments from the Buffalo River have been reported at 3 $\mu g\ g^{-1}$ for BaP, 13 $\mu g\ g^{-1}$ for fluoranthene, and 17 $\mu g\ g^{-1}$ for pyrene (Black, 1983b). Similarily, concentrations of these compounds in sediments from the Detroit River have been reported to range from 0.12 to 17.6 $\mu g\ g^{-1}$ for BaP, 0.12 to 38.8 $\mu g\ g^{-1}$ for pyrene, and 0.12 to 34.6 $\mu g\ g^{-1}$ for fluoranthene (Fallon and Horvath, 1985). These sediment concentrations are similar to those reported for contaminated areas in Puget Sound, Washington (Krahn et al., 1986), and average BaP equivalents in most fish examined in this study were equal to or greater than those reported for English sole from Puget Sound (Krahn et al., 1986). Thus, fish from the Buffalo and Detroit Rivers are potentially exposed to high levels of multi-ringed aromatic compounds that reflect the metabolite profiles in the bile. Moreover, chromatographic profiles were similar to those

observed in the bile of bullheads that had been fed BaP in laboratory studies (Maccubbin et al., 1988).

Levels of adducts in liver and BaP equivalent levels in bile were both elevated in wild bullheads relative to laboratory-raised bullheads, but were not linearly correlated. This is probably because adduct levels are the result of exposure to chemicals over a period of time, while the BaP equivalents in bile reflect sediment, dietary, or aqueous exposures that are relatively recent. Microcosm studies have demonstrated that sediment-bound BaP is bioavailable to fish and is metabolized rapidly (Varanasi et al., 1985). In addition, laboratory studies with brown bullheads and other fish species have demonstrated that within 24 h of feeding with BaP, metabolites can be found in the bile (Vetter et al., 1985; Maccubbin et al., 1988).

Although brown bullheads from the Buffalo River had the highest prevalence of liver tumors, the adduct levels in bullheads were lower than those in carp and white suckers from the Buffalo River. The lack of appropriate reference fish for species other than bullheads makes inter-specific comparisons difficult. Possibly, adduct levels observed in walleye, carp, and suckers are normal in these species, with autoradiograph spots representing background levels of DNA adducts analogous to those reported in the liver of rats (Gupta et al., 1982; Randerath et al., 1981).

Species differences in susceptibility to tumor formation (for example, due to differences in repair of DNA damage), and environmental factors that affect promotion and progression of neoplastic cells may vary the carcinogenic response to DNA adduct formation. The prevalences of neoplasia in bullheads, white suckers, and carp vary greatly. There have been many reports of liver tumors in brown bullheads (Dawe et al., 1964; Brown et al., 1973; Baumann et al., 1982; Black, 1983b; Couch and Harshbarger, 1985). Liver neoplasia has also been reported in white sucker (Dawe et al., 1964; Dawe et al., 1976; Hayes et al., this volume). To our knowledge, no reports of liver tumors exist for carp. All of these fish species may be exposed to the same sediment-bound chemicals while cohabiting polluted environments in the Great Lakes region. However, even though genetic damage to liver cells has occurred, as evidenced by DNA adduct formation, tumors need not always result. Although initiation by chemical binding to DNA is a necessary step in chemical carcinogenesis, the overall importance of this process to tumor development in fish is still being studied.

At present, the ^{32}P-postlabeling assay alone is not sufficient to demonstrate that exposure to sediment-bound chemicals is the cause of tumors observed in wild fish. However, the addition of this assay into an integrated study using other methods, such as HPLC analysis of bile, and histopathology, can provide the researcher with a powerful battery of analytical techniques to investigate the role of chemical contaminants in the induction of fish tumors. The results of these preliminary studies point out the need for more detailed research into the steps involved in carcinogenesis, mechanisms of DNA repair, metabolism of xenobiotics, and bioavailability of sediment-bound chemicals in a variety of fish species. The ^{32}P-postlabeling technique may be a useful tool for studying the metabolism of xenobiotics to DNA-reactive species, the kinetics of DNA adduct formation, and the persistence of these adducts in various fish tissues. Finally, archived tissue samples could be analyzed to examine historical trends in chemical exposure to fish. Given the wide applications and extreme sensitivity of the method, it is likely that the ^{32}P-postlabeling technique will become widely used by researchers investigating carcinogenesis in fish.

ACKNOWLEDGEMENTS

This research was supported in part by Cooperative Agreement CR812575-02-0 with The United States Environmental Protection Agency and by Grant A3403 from The Natural Sciences and Engineering Research Council, Canada. Additional support was provided by an International Cancer Research Technology Transfer award to AEM from the International Union Against Cancer. We would like to thank Mrs. Judith Vangalio for excellent technical assistance and manuscript preparation.

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The Science of the Total Environment, 94 (1990) 105–123
Elsevier

PATHOGENESIS OF SKIN AND LIVER NEOPLASMS IN WHITE SUCKERS FROM INDUSTRIALLY POLLUTED AREAS IN LAKE ONTARIO

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ABSTRACT

Increased prevalences of epidermal and hepatobiliary neoplasms in white suckers(Catostomu commersoni) and brown bullheads (Ictalurus nebulosus) in the Western region of Lake Ontario have been associated with industrial pollution, but the identity and causative role of environmental carcinogens have not yet been established. Most epidermal tumors of lip and body skin are benign focal proliferations that occur in fish from the polluted Hamilton region, and also in fish from less polluted sites in the Great Lakes. These skin tumors in white suckers do not have consistent alterations in cellular glutathione S-transferases (GST), suggesting that growth of skin tumors is not promoted by chemicals normally detoxified by GST. However, elevated levels of glutathione peroxidase (GPO) and glutathione reductase (GR) in skin papillomas are indicative of promotional peroxidative tissue injury, either caused directly by xenobiotics or indirectly by chemical-induced inflammation. Liver tumors in white suckers from Lake Ontario include preneoplastic, benign, and malignant populations of hepatocellular and biliary cells, all of which are more prevalent in fish from polluted sites. These liver tumors

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are consistently associated with chronic cholangiohepatitis and segmental cholangiofibrosis, but these conditions also occur in white suckers in non-industrial locations. Thus, the natural occurrence of biliary disease, not attributable to industrial pollution, may have some influence on the development of liver tumors. Some preneoplastic lesions and the majority of neoplastic hepatocellular and biliary lesions in white suckers have low levels of total GST, indicating that these liver neoplasms are not promoted by xenobiotics normally detoxified by hepatic GSTs. These studies suggest that an understanding of the biochemical changes and pathogenesis of tumors in white suckers can serve as a basis for identifying the chemicals involved in the initiation, promotion, and progression of these skin and hepatic neoplasms.

INTRODUCTION

The widespread recognition of increased rates of skin and hepatic neoplasms in wild fish inhabiting polluted marine or fresh water environments (Sonstegard, 1977; Smith et al., 1979; Black, 1983; Mix, 1985; Couch and Harshbarger, 1985; Kranz and Peters, 1985; Malins et al., 1987; Myers et al., 1987) are the basis for two important hypotheses. First, it is reasonable to predict that some xenobiotics in polluted sediments play a role in the initiation, promotion, or progression of the neoplasms observed (Baumann et al., 1982; Black, 1983; Black, 1984; Kranz and Peters, 1985; Peters et al., 1987; Metcalfe et al., this volume ; Myers et al., this volume). If this is true, wild fish may be useful sentinels for aquatic pollution by carcinogenic chemicals (Sonstegard, 1977; Sonstegard and Leatherland, 1984; Mix, 1985; Couch and Harshbarger, 1985), and may provide a direct epizootiological indicator of excessive pollution or successful "clean up" strategies. The second important hypothesis is that the affected fish are similarly susceptible to xenobiotics also carcinogenic to humans and other terrestrial mammals, implying that industrial generation of the carcinogens responsible is an avoidable occupational hazard for workers in the industrial sources of the pollution.

Several pieces of evidence have a direct bearing on the reliability of these hypotheses. First, the pathogenesis of neoplasia is a complex process (Farber and Sarma, 1987) involving multiple interacting factors (eg. toxicants, diet, infectious diseases, etc.) some of which might be specific for an affected species in a polluted ecosystem. If this is true, the increased occurrence of neoplasms might simply be a non-specific result of the general perturbations in a polluted aquatic environment, rather than a response to specific carcinogenic chemicals. Second, some fish in these polluted environments seem to be more resistant to tumor induction. For example, the common carp (*Cyprinus carpio*) in the Great Lakes evidently does not develop the same high prevalence of neoplasms as observed by Smith and Ferguson (1985) for white suckers (*Catostomus commersoni*) and brown bullheads (*Ictalurus nebulosus*). The starry flounder (*Platichthys stellatus*) does not exhibit the same prevalence of neoplasia as do English sole (*Parophrys vetulus*) in Puget Sound (Malins et al., 1987; Myers et al., 1987). This suggests the need for more information on the relative susceptibility of different species of fish to carcinogens, if the occurrence of neoplasms in some species is to be used to detect human carcinogens in the environment or in the industrial sources of the associated pollution.

We (Smith and Ferguson, 1985; Smith et al., in press), and others (Sonstegard 1977) have observed a higher frequency of skin and hepatic neoplasms in white suckers and brown

bullheads from the polluted Hamilton region of western Lake Ontario than from less polluted sites in Lakes Huron, Simcoe and Erie. Our approach to evaluating the hypothesis that fish are sentinels for human carcinogens has been to characterize the pathogenesis of these neoplasms as a means of identifying: a) species-specific intercurrent disease processes which might contribute to tumor development, b) the pathogenetic mechanisms of tumor development as a means of determining if genotoxic initiating carcinogens or non-genotoxic promoting toxicants are involved in tumor development, c) whether the affected fish are more or less susceptible to genotoxic carcinogens than unaffected species of fish in the same locations, and d) whether the biochemical basis of carcinogen susceptibility of affected fish is equivalent to that in terrestrial mammals. In this report, we present our accumulated epizootiological, pathologic and biochemical evidence and discuss the relevance of this data to the major hypotheses stated above.

METHODS AND MATERIALS

Mature white suckers were collected during their spawning migrations into rivers and streams entering various sites in the Great Lakes. Some suckers were collected from Oakville and Grindstone Creeks near Hamilton, a heavily polluted industrial region of western Lake Ontario, Canada. Reference areas were non-industrial locations, including less polluted sites in Georgian Bay, in Lake Huron (Keefers Creek), and in Lake Simcoe (Whites Creek), Ontario. Brown bullheads were captured in summer in the polluted area of Hamilton Harbour, or from Long Point Bay, Ontario on Lake Erie, a non-industrialized, less polluted reference site. Most fish were kept briefly in laboratory tanks until necropsy examination, mostly within one week of capture. In studies on the reversal of skin tumors, some white suckers were kept captive for 3 mo to monitor progressive changes in skin tumors in a clean environment. Fish were killed with an overdose of anesthetic for necropsy, examination, and tissue collection.

Pathological Studies

External skin surfaces were examined for proliferative lesions. Skin tumors on lips and body skin, and unaffected skin samples were collected. Some tissues were processed for biochemical analysis, as described below, but the majority were fixed in Bouin's (scaled skin) or 10% neutral buffered formalin (lip skin) for routine histopathological examination. Some samples were fixed in 4% glutaraldehyde for ultrastructural examination. Sections were routinely stained with hematoxylin and eosin (H&E), or by the immunoperoxidase technique (see below).

Liver samples from grossly normal or nodular livers were also collected and processed as above for histopathological examination or biochemical analysis.

Biochemical Studies

Samples of lip papillomas, unaffected adjacent lip tissue, or lip tissue from unaffected fish were collected on ice, and then homogenized with a Polytron tissue grinder (Brinkman) in 3 volumes of cold 0.25 M sucrose, containing 1 mM EDTA, 25 mM HEPES (pH 7.4),

and 1 mM dithiothreitol. Homogenates were centrifuged at 10,000g (30 min, 4°C) and the supernatant was further centrifuged at 100,000g (60 min, 4°C) to yield a clear supernatant (cytosol) which was frozen at -70°C until assayed further. Normal liver tissue and liver tumors were homogenized in a glass-teflon homogenizer, but otherwise were processed as for skin samples above.

Protein determinations were performed by a modification of the Lowry method. Cytosolic glutathione S-transferase (GST) activities were determined (at pH 7.0, 25°C) by the method of Habig et el. (1974) with 1-chloro-2,4-dinitrobenzene (CDNB). Glutathione reductase (GR) activity was determined at 25°C in a reaction mixture consisting of 0.5 mM oxidized glutathione, 0.1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.5) with 1% Triton-X-100. Glutathione peroxidase (GPO) activity was determined at 25°C in a reaction mixture of 0.4 μM t-butyl peroxide, GR (0.3 U mL^{-1}; Sigma), NADPH (0.25 mM) and reduced glutathione (5.2 mM) in 0.06 M sodium phosphate buffer (PH 7.5) and 1% Triton-X-100. Activities of all these enzymes were monitored kinetically at 340 nm, and expressed per mg cytosolic protein or per mg of wet tissue.

Samples of cytosol were separated by SDS-polyacrylamide (15%) slab gel electrophoresis (SDS-PAGE) under reducing conditions by the methods described by Rushmore et al. (1987) and Rushmore et al. (1988). Cytosol from grossly normal livers of white suckers was further purified by S-hexylglutathione Sepharose affinity chromatography (Ramage and Nimmo, 1983; Mannervik et al., 1985). Affinity purified hepatic GSTs were used as antigens to immunize rabbits to prepare polyclonal antiserum to normal hepatic GST isoenzymes by methods similar to those used for immunizing rabbits against rat GSTs (Rushmore et al., 1988). The anti-GST antiserum reacted specifically with the three major bands of hepatic GST from white suckers, as determined by Western blot analysis of hepatic cytosol separated on SDS-PAGE gels (Rushmore et al., 1988).

Immunocytochemistry

Formalin-fixed sections of liver and skin from white suckers with or without tumors were trypsinized and stained by the peroxidase-antiperoxidase (PAP) method for GST, using commercial kit reagents (Dako Corporation, Santa Barbara, California). Rabbit anti-sucker GST at various dilutions was used as the primary specific antibody. Color reaction was developed by incubation with diaminobenzidine-4HCl and hydrogen peroxide. Specific staining was defined as a positive brown staining observed only in the presence of specific anti-GST antiserum. Because of the low incidence of hepatocellular tumors in these surveys, we also stained additional formalin-fixed samples of hepatocellular neoplasms from other locations supplied by D. Rokosh (Ontario Ministry of the Environment, Rexdale, Ontario, Canada) and V. Cairns (Canadian Centre for Inland Waters, Burlington, Ontario, Canada).

DNA Binding

The ability of hepatic cytosols or affinity purified GSTs to inhibit DNA binding by rat-microsome activated [^{3}H]-aflatoxin B_1 was assayed according to the method of Lotlikar et al., (1984). Unlabeled aflatoxin B_1 was obtained from Makor Chemicals, Jerusalem,

Israel. Washed rat hepatic microsomes were prepared by the method described by Roomi et al. (1985).

Sequential Changes in Skin Neoplasms

We evaluated the influence of the native environment on the development of lip and skin neoplasm of white suckers from Oakville Creek. Fifty wild white suckers were tagged and examined for lip and skin papillomas at the time of capture and maintained for up to 3 mo in "clean" laboratory holding tanks serviced by ground water (10°C). The size, character, and location of all skin lesions were recorded only after the 3 mo holding period.

RESULTS

Occurrence of Neoplasms

White suckers from Lake Ontario streams had more skin and hepatic neoplasms than similar-sized fish from the less-polluted reference sites in Ontario, Canada, namely Georgian Bay, Lake Huron, and Lake Simcoe (Figure 1). Similar skin and liver neoplasms were also observed in brown bullheads from Hamilton Harbour in western Lake Ontario

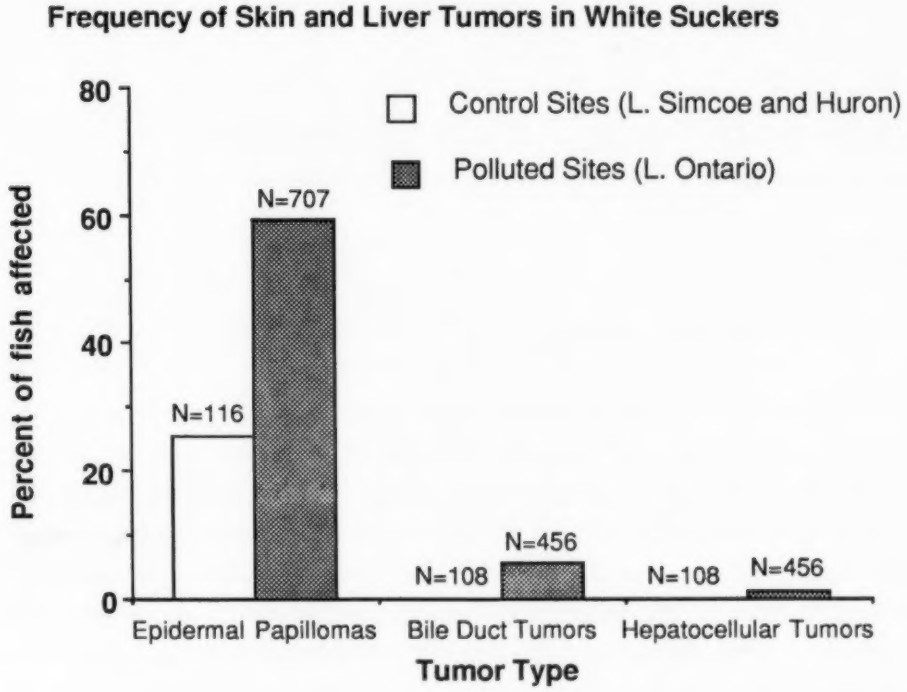

Figure 1. **Frequency of skin and liver tumors observed in white suckers captured from Oakville and Grindstone Creeks, Lake Ontario, in comparison with white suckers from less polluted Whites Creek, Lake Simcoe, and Keefers Creek, Lake Huron, Ontario. Hepatocellular tumors refer to lesions greater than 2 mm. Fish with smaller altered foci were excluded.**

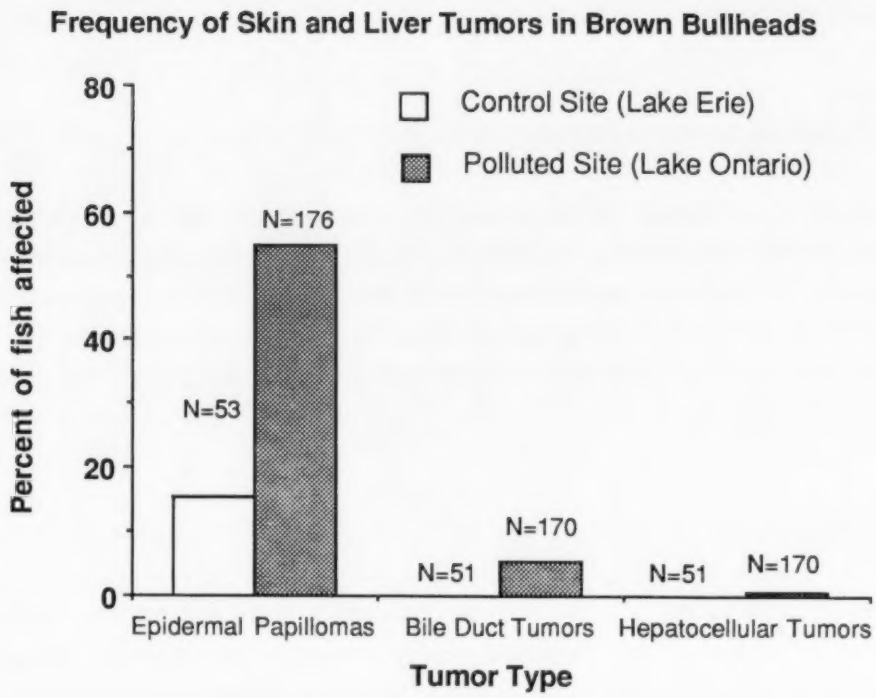

Figure 2. **Frequency of skin and liver tumors in brown bullheads from Hamilton Harbour in comparison with fish from a less polluted site (Long Point Bay, Lake Erie).**

or Long Point Bay in Lake Erie (Figure 2). These tumors in brown bullheads have not yet been studied to the extent described below for white suckers.

Pathological Findings

Epidermal neoplasms of white suckers

On white suckers, skin tumors occurred most frequently on the lips, but also affected were the body skin and fins. We use the term papilloma as a general descriptive name for these lesions, even though some are more plaque-like in gross appearance. Typically, the papillomas were focal proliferations of epidermal cells, resulting in marked expansion of malpighian keratinocyte populations, displacement of epidermal mucous cells, and frequent marked folding of the basal cells into finger-like projections extending into the underlying dermis. This infolding was usually associated with a local leukocyte and neutrophil inflammatory response. Very rarely (1.4%), the degree of local dermal extension was sufficiently severe to warrant terming lesions as squamous cell carcinomas, solely on the basis of local invasion. In our surveys, frank malignancy with local or regional metastasis has not been observed in epidermal neoplasms of white suckers (Smith et al., in press). Viruses were not identified in any papillomas examined ultrastructurally (Smith et al., 1989). The histological appearances of these epidermal lesions are generally similar to those described in other species of fish with naturally occurring epidermal proliferative lesions (Smith et al., 1987a).

Liver neoplasms in white suckers

Liver tumors were more variable in gross and histological appearance than were epidermal tumors. The number of large grossly visible tumors was relatively low, and not all were neoplasms. Many small tumors were identifiable only by microscopic examination of liver sections. We infrequently observed discrete spherical white hepatic nodules which had the histological features of inflammatory granulomas that we attribute to migratory helminth parasites. Hepatic neoplasms were recognizable as either hepatocellular or biliary in cell type, but some of the more advanced tumors were heterogeneous and not reliably categorized by cell type. Hepatocellular lesions ranged from small (>200 μm) round or irregular foci to larger grossly-visible nodules or tumors of phenotypically altered hepatocytes (Figure 3). The range of altered hepatocyte populations observed included basophilic and clear cell types resembling spontaneous hepatocellular foci and neoplasms in other wild fish (Myers et al., 1987; Peters et al., 1987) and lesions induced by carcinogens in rodents (Farber and Sarma, 1987) and in fish (Hendricks et al., 1984; Nakazawa et al., 1985; Bailey et al., 1987). Larger hepatocellular neoplasms were expansive, or often locally invasive within the liver (Figure 4). These lesions were composed of polygonal cells with anatomic features most closely resembling normal hepatocytes, but with a higher nucleus to cytoplasmic ratio, and less cytoplasmic differentiation. We did not observe foci or neoplasms with the eosinophilic cytoplasmic differentiation typical of the "resistant" nodule

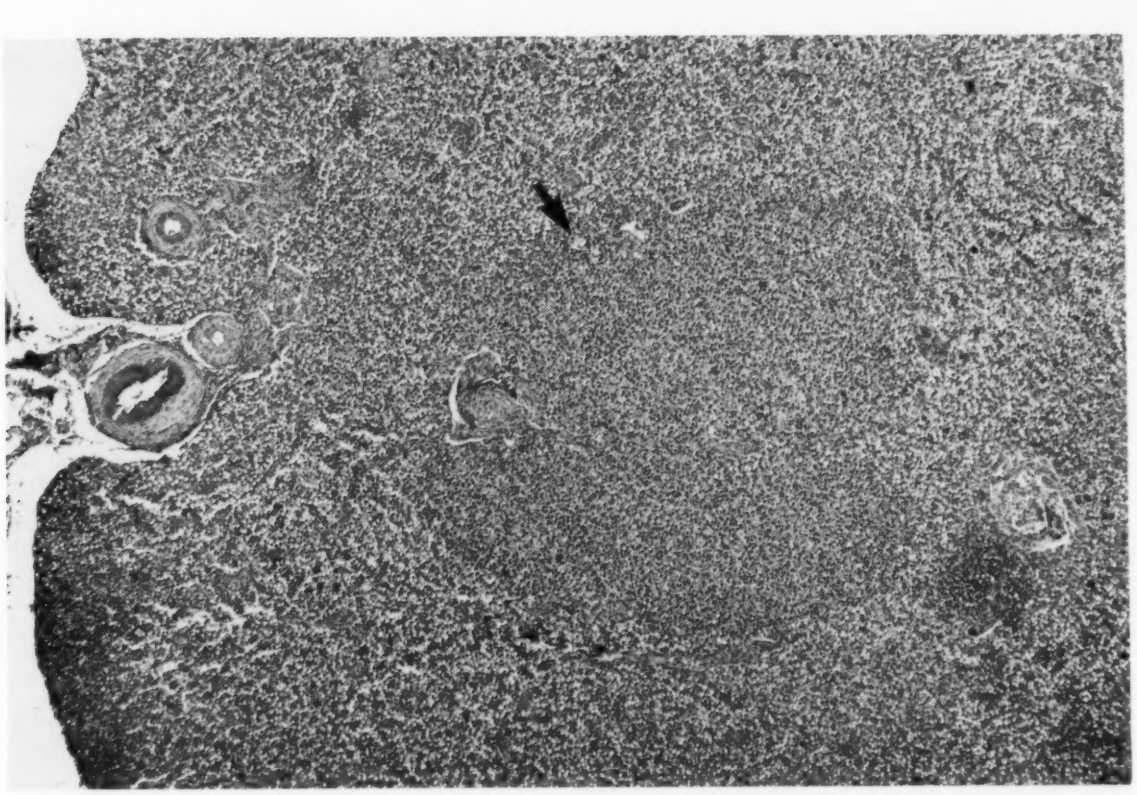

Figure 3. Discrete focus of phenotypically altered (basophilic) hepatocytes (arrow) in a section of liver from a white sucker from Lake Ontario. These altered hepatocyes are typically more basophilic with less cytoplasmic vacuolation than surrounding normal liver (H & E, X 40).

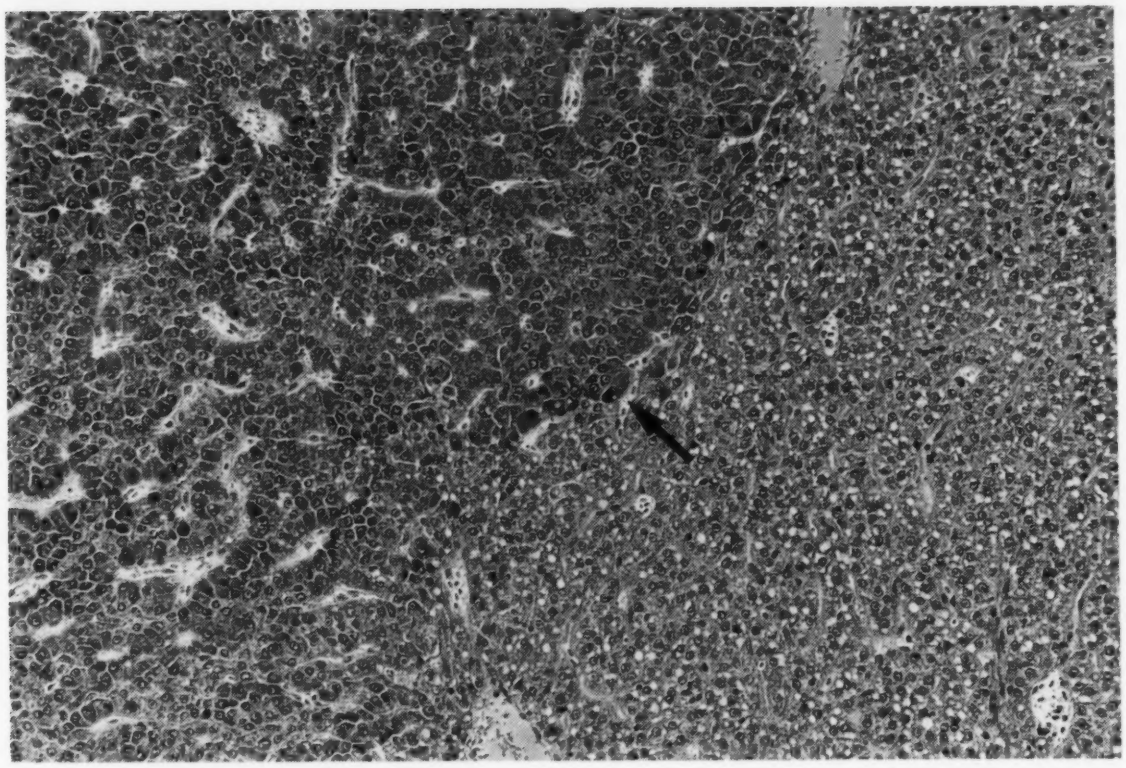

Figure 4. The arrow indicates the edge of a locally invasive trabecular hepatocellular carcinoma in a section of liver from a white sucker from Lake Ontario. (H & E X 100)

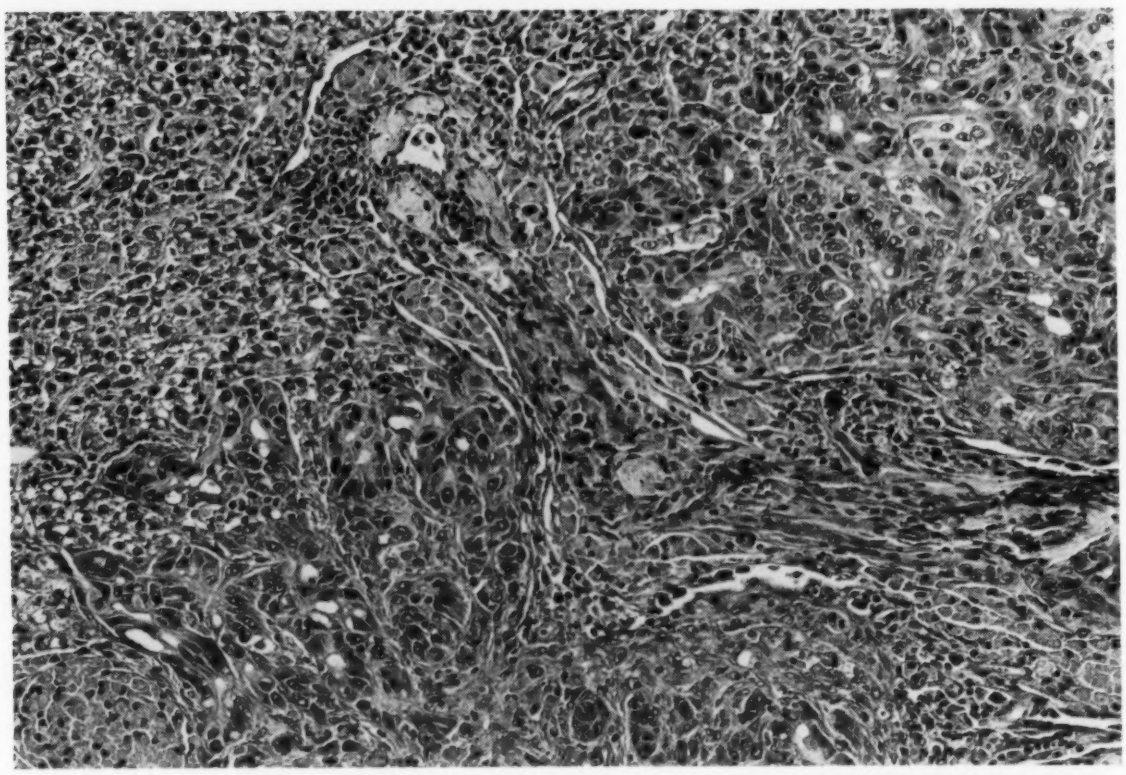

Figure 5. Locally invasive bile duct carcinoma in a section of liver from a white sucker from Lake Ontario. (H & E X 200).

phenotype (Farber and Sarma, 1987). In Figure 1, the number of fish with hepatocellular tumors refers to those with large hepatocellular lesions and does not include fish with only altered foci less than 2 mm in diameter in sections.

As illustrated in Figure 5, biliary neoplasms were either discrete nodules of irregularly arranged ducts within a connective tissue matrix (termed bile duct adenomas) or extensively invasive prolific tumors composed of less well-differentiated irregular duct-like structures (termed, bile duct carcinomas). Bile duct tumors were more frequent than hepatocyte neoplasms in white suckers from Lake Ontario.

In most white suckers, especially those from Lake Ontario, we observed two distinct proliferative reactions of intrahepatic bile ducts. The most frequent change occurred along major intrahepatic portal tracts and was typified by hyperplastic biliary epithelium frequently folded into lumenal projections many cells thick, and an associated marked periductular inflammatory reaction. This local inflammatory process was chronic and usually fibrotic, accompanied by a significant increase in mononuclear inflammatory cells (Figure 6). We term this diffuse hepatobiliary disease cholangiohepatitis. We also observed focal segmental proliferations of cholangioles, accompanied by fibroplasia, atrophy of hepatocytes and enlargement of melano-macrophage nodules. These lesions, which we term segmental cholangiofibrosis, were usually located at the periphery of hepatic lobes and apparently result from focal bile duct obstructions. Cholangiohepatitis and cholangiofibrosis

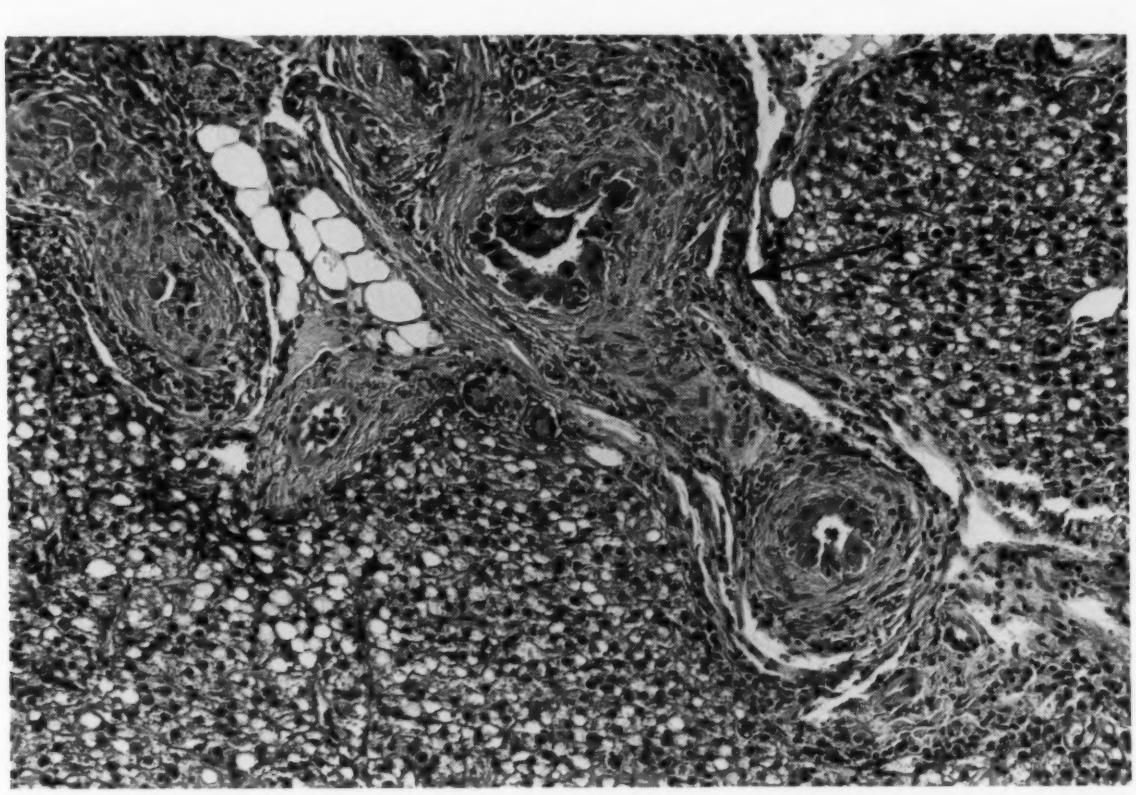

Figure 6. Chronic inflammatory reaction surrounding hyperplastic bile ducts in the liver of a white sucker from Lake Ontario. Note the increased fibrous connective tissue and numerous mononuclear inflammatory cells (arrow) around the bile ducts, and the hyperplastic duct epithelial cells, some of which are exfoliating. (H & E section X 100).

Table 1. Frequency of proliferative hepatobiliary disease (cholangiohepatitis and cholangiofibrosis) in white suckers and brown bullheads from Lake Ontario in comparison with fish from less polluted control sites in Lake Huron, Lake Simcoe and Lake Erie.

	Percent of fish affected	
	White Suckers	Brown Bullheads
Control sites		
Whites Creek (L.Simcoe)	73 (71)[a]	-
Keefers Creek (L.Huron)	29 (37)	-
Long Point Bay (L.Erie)	-	33 (51)
Polluted sites		
Grindstone Creek (L.Ontario)	67% (147)	-
Oakville Creek (L.Ontario)	77% (309)	-
Hamilton Harbour (L.Ontario)	-	36% (170)

[a]Values in parentheses are the total number of fish examined histologically.

were most severe in Lake Ontario fish with concurrent hepatocellular or biliary neoplasia. However, less severe cholangiohepatitis and/or cholangiofibrosis without neoplasia were evident at a similar or lower frequency in white suckers from the less polluted reference sites (Table 1). Brown bullheads were similarly affected with cholangiohepatitis and cholangiofibrosis (Table 1). Because these biliary proliferative reactions were frequently focally severe and associated with small expansive cholangiomas in Lake Ontario fish, it is possible that some of these focal proliferations represent preneoplastic altered biliary epithelium (analogous to altered hepatocyte foci). This hepatobiliary proliferative disease is similar to lesions described in English sole (Myers et al., 1987), and may be related to helminth parasitism in the liver.

Biochemical Studies

Lip skin papillomas in white suckers

Lip papillomas had increased activity of cytosolic GST compared with lip skin from unaffected fish (Table 2). However, GST activity was similarly elevated in surrounding lip skin (Table 2). By comparison, activities of GR and GPO were substantially elevated in lip papillomas in comparison with normal or surrounding lip skin (Table 2). SDS-PAGE analysis of cytosolic proteins from lip papillomas revealed that these tumors had slightly

Table 2. Activities of glutathione S-transferase (GST), glutathione peroxidase (GPO) and glutathione reductase (GR) in normal lip and in lip papillomas and surrounding skin of white suckers (*Catostomus commersoni*) from Lake Ontario.

Enzyme	Normal (19)	Tumors (6)	Surrounding (6)
GST[a]	25 ± 10	41 ± 12*	47 ± 33
GPO[b]	53 ± 21	96 ± 51*	61 ± 38
GR[c]	11 ± 4	38 ± 4*	14 ± 10

[a]CDNB conjugation (μmol/min/mg cytosolic protein) determined at pH 7.0 at 25°C (Habig et al. 1974).

[b]NADPH utilized (μmol/min/mg cytosolic protein) determined at 25°C in a reaction mixture also containing t-butyl peroxide, glutathione reductase, and reduced glutathione (pH 7.5).

[c]NADPH utilized (μmol/min/mg cytosolic protein) determined at 25°C in a reaction mixture also containing oxidized glutathione (pH 7.5).

*Significantly different by one-way analysis of variance ($p \leq 0.05$)from normal skin values.

greater amounts of GST subunit bands than did normal skin. However, the number and distribution of subunit bands in papillomas were similar to those of normal lip skin or surrounding skin (not shown). The amount of GST in skin samples was much lower than in liver, a finding confirmed by very weak or negative immunocytochemical staining for GST in papillomas and lip epidermis (see below).

Analysis of hepatic GST in white suckers

Normal liver contained high levels of GST activity as determined by CDNB conjugation rates (Table 3). Male fish had about twice the activity as female fish (Table 3), a finding that correlated with the reduced ratio of cytoplasm to nucleus volume in hepatocytes of spawning female white suckers (not shown). We observed lower hepatic GSTs in male fish with lip papillomas, but the significance of this finding is unknown. SDS-PAGE profiles of

Table 3. Activities of glutathione S-transferase (GST) in hepatic cytosol from white suckers (*Catostomus commersoni*) from Lake Ontario.

	GST activity (μmol CDNB/min/gram liver)	
	Males	Females
Controls (without lip papillomas)	18.2 ± 5.6* (N = 13)	8.2 ± 2.5 (N = 12)
Affected (with lip papillomas)	11.9 ± 2.5 (N = 3)	9.0 ± 0.6 (N = 3)

[a]Determined at pH 7.0 at 25°C (Habig *et al.*, 1974).

*Significantly greater than female values ($p \leq 0.05$) by one way analysis of variance

hepatic cytosol revealed one minor and three major subunit bands in the 25-28 kD region (Figure 7). These four subunits were present in SDS-PAGE profiles of hepatic cytosol proteins eluted with S-hexyl glutathione from S-hexylglutathione-sepharose affinity columns (Figure 7).

In the two hepatic tumors that were large enough for analysis, no new GST bands were recognized because the total amount of GST protein in these cytosol preparations was much lower than observed in whole liver. For this reason we chose to further characterize GST levels in hepatic tumors by immunocytochemical methods.

Our preparation of rabbit anti-GST, prepared by immunizing rabbits with affinity-purified GST from normal liver of white suckers, specifically recognized three major subunits of affinity-purified hepatic GST by Western blot analysis and did not cross react with other bands in whole cytosol. This antiserum resulted in strong specific immunoperoxidase staining of hepatocytes, renal proximal tubular epithelium, gill epithelium and the epithelium of major intrahepatic bile ducts. All bile duct adenomas and carcinomas had almost undetectable GST staining in comparison with hepatocytes in the same liver. The majority of altered hepatocyte foci had normal GST but some foci and all nodules and hepatocellular neoplasms had much weaker GST staining in comparison with surrounding liver. No hepatic neoplasm with increased (induced) immunoreactive GST was found.

Effects of hepatic GST on DNA binding by AFB_1

Affinity purified hepatic GSTs from white suckers had a concentration- dependent ability

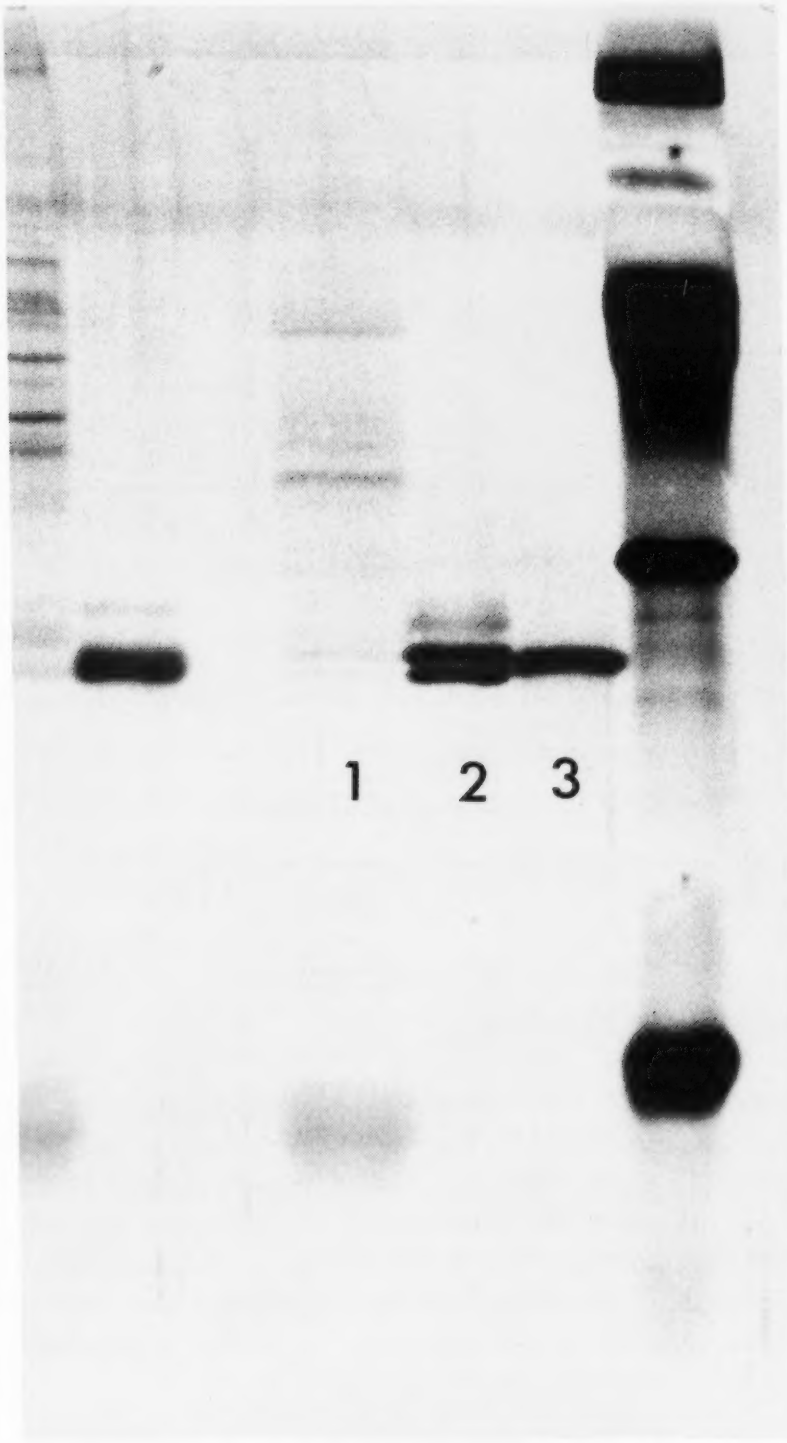

Figure 7. SDS-PAGE profiles of cytosol (1) and affinity purified glutathione S-transferases (2) from the liver of a white sucker. Purified rat glutathione S-transferase-P (subunit MW=26 KD) is shown in lane 3.

to inhibit rat-microsome activated AFB_1 binding to calf thymus DNA (Figure 8). This capacity to inhibit AFB_1 binding was similar to that of hepatic GSTs affinity purified from mouse liver cytosol. This indicates that normal hepatic GSTs of white suckers are able to detoxify AFB_1, similar to hepatic GSTs from the mouse, a mammalian species highly resistant to the hepatocarcinogenic effects of AFB_1.

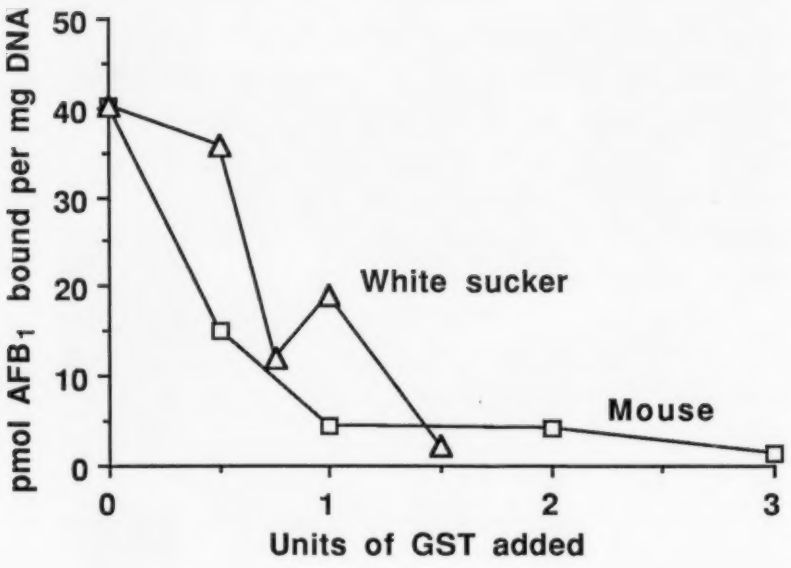

Figure 8. Inhibition of aflatoxin B_1 (AFB_1) binding to DNA by affinity purified hepatic glutathione S-transferases (GST) from white suckers. For comparison, inhibition by GST from a mouse, a species resistant to AFB_1, is shown. Assays were conducted according to Lotlikar et al. (1984), using rat microsomes to activate AFB_1 and 5 mM reduced glutathione with GST to block binding to calf thymus DNA.

Sequential Changes in Skin Papillomas

Many papillomas on the lips and body skin of white suckers captured from Lake Ontario (Oakville Creek) disappeared during a 3 mo period of captivity in "clean" laboratory tanks. However, it was observed that some new skin tumors developed in these captive fish. These observations suggested that development of papillomas did not require factors present in polluted environments. Histological studies of skin papillomas in white suckers indicated that these lesions were associated with a significant local inflammatory reaction, similar to that observed in skin papillomas caused by viruses in mammals.

DISCUSSION

The prevalence of skin papillomas in white suckers and brown bullheads from polluted regions of the Great Lakes is greater than in less polluted sites, as demonstrated in this study and in several independent studies (Sonstegard, 1977; Black 1983; Black, 1984; Baumann et al., 1982; Smith and Ferguson, 1985). Skin papillomas on white suckers often disappeared when fish were removed to a laboratory environment supplied with ground water. There is some evidence that the tumours also persist on fish in the wild, or on fish kept in tanks supplied with Lake Ontario water (V. Cairns, personal communication). This implies that some environmental factor is involved in the development of these lesions.

However, recent findings suggest that environmental carcinogens are not solely responsible for the pathogenesis of these skin tumors. The fact that these lesions on white suckers and brown bullheads occur widely in the Great Lakes system implies that the initiating and promoting influences involved in papilloma development are also similarly widespread. The evidence that some new papillomas develop on fish maintained in a clean environment suggests that growth of these lesions does not require an environmental chemical promoter (Smith and Zajdlik, 1987). So far, we have not been able to identify a virus (Smith et al., 1989) as a possible cause of these lesions.

One obvious possibility is that the environmental chemicals enhance the development of papillomas initiated by another cause. Application of chemicals can initiate or promote the development of papillomas and carcinomas in rodents (Slaga et al., 1980; Hennings et al., 1983). Topical application of genotoxic carcinogens causes a high rate of conversion of mouse papillomas to aggressive squamous cell carcinomas (Hennings et al., 1983). In white suckers and brown bullheads, we rarely observed conversion of papillomas to malignant skin tumors, and those lesions classified as squamous cell carcinomas had limited aggressive behaviour. This suggests that papillomas on fish from Lake Ontario are not subject to continuous genotoxic insults. Lip papillomas in brown bullheads captured from some industrial sites that are heavily polluted by polycyclic aromatic hydrocarbons (PAHs) do exhibit a high rate of conversion to malignancy (Baumann et al., 1982), and topical application of extracts from contaminated sediments can induce papillomas on brown bullheads (Black, 1983). Because lip papillomas in white suckers have induced activities of GST, they might be more resistant to the continued activity of PAHs, and paradoxically, less likely to be converted to malignant carcinomas. These various observations are consistent with a hypothesis that the polluted aquatic environment and sediments can influence the growth of skin papillomas in susceptible fish to some extent, but may not be required for their development.

Our observations of subjacent leukocytic inflammation in papillomatous skin (Smith et al., in press), and elevated GPO and GR activities in lip papillomas collectively suggest that papillomas might be promoted by peroxidative mechanisms, possibly of leukocyte origin. The inflammatory reactions are perhaps an indication of local irritation or immune responses to infectious agents in these lesions. So far we have not been able to determine the pathogenesis of such an inflammatory process associated with epidermal papillomas in white suckers.

Our observations on GST levels and functions in white suckers indicate that this class of detoxification enzymes provides an important mechanism of cellular resistance to xenobiotics, especially in the liver, kidney, and gills of these fish. Thus, white suckers resemble mammals (Mannervik, 1985; Mannervik et al., 1985) and other species of fish in which GST functions have been examined (Nimmo, 1987). Hepatic GST from white suckers did protect DNA against covalent binding of AFB_1 metabolites, suggesting that hepatic GSTs may be an important defence against some genotoxic carcinogens in white suckers. Further studies are required to fully evaluate the capacity of GST isoenzymes of these fish to prevent DNA damage by important environmental carcinogens such as PAHs, which are significant industrial pollutants in Hamilton Harbour (Harlow and Hodson, 1988).

We postulated that hepatic and skin neoplasms of white suckers would have elevated levels of GST isoenzymes if they were promoted by xenobiotics normally detoxified by constitutive or inducible GST isoenzymes. In some models of chemically promoted

hepatocarcinogenesis in rodents (Farber and Sarma, 1987), initiated preneoplastic foci of hepatocytes with elevated GST activity, due mainly to an inducible isoenzyme (GST-P), are readily promoted to nodules and carcinomas that express the GST-induced phenotype (Eriksson et al., 1983; Sato et al., 1984; Satoh et al., 1985; Tatematsu et al., 1985; Rushmore et al., 1987; Rushmore et al., 1988). However, we did not find elevations of GST in any preneoplastic or neoplastic proliferations of hepatocytes or bile duct epithelium in white suckers. This suggests that the development of these liver lesions is not due to promotion by resistance to xenobiotics detoxified by GSTs in the manner described in the resistant hepatocyte or inducer promoting rodent models. It is not yet known if the lesions in white suckers are resistant to xenobiotics by virtue of loss of cytochrome P_{450}-mediated activation capacity, a form of resistance also observed in many chemically promoted liver tumors of rodents (Roomi et al., 1985; Farber and Sarma, 1987). Also, promotion of liver tumors by xenobiotics cannot be ruled out because our observations of normal or reduced GST content in liver tumors are similar to those in studies of hepatocarcinogenesis in rodents exposed to peroxisomal inducers (Rao et al., 1986). In fish from other polluted sites, a higher incidence of preneoplastic hepatocellular lesions, especially of the eosinophilic type, has been observed (Myers et al., 1987), and might be analogous to the resistant-hepatocyte rodent models.

In western Lake Ontario, bile duct neoplasms were more frequent than hepatocellular neoplasms and were associated with proliferative biliary diseases (cholangiohepatitis and cholangiofibrosis) that were less severe in fish from our reference sites. It is reasonable to expect that the proliferative bile duct epithelial changes would predispose fish to initiation and promotion of bile duct neoplasia, especially in individuals exposed to higher levels of carcinogens excreted in the bile. For this reason, we expect that an understanding of the detoxification and excretory mechanisms of these fish and the identification of carcinogenic biliary metabolites (Varanasi et al., 1987) is a reasonable basis for comparing the sensitivity of the affected fish with other species of fish and terrestrial mammals. However, the evidence from our studies suggests that bile duct neoplasia is a multifactorial disease that cannot be explained solely on the basis of exposure to environmental carcinogens.

ACKNOWLEDGEMENTS

This work was supported by the Ontario Ministry of Environment, the Natural Sciences and Engineering Research Council of Canada, the World Wildlife Fund and the Medical Research Council of Canada.

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The Science of the Total Environment, 94 (1990) 125–141
Elsevier

CARCINOGENIC AND GENOTOXIC ACTIVITY OF EXTRACTS FROM CONTAMINATED SEDIMENTS IN WESTERN LAKE ONTARIO

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ABSTRACT

In order to investigate the putative association between chemical contamination in western Lake Ontario and high prevalences of fish tumors, sediments from Hamilton Harbour and Oakville Creek in Lake Ontario and reference sites in non-polluted areas of Ontario, Canada were collected and extracted for organic contaminants. Sediment extracts from Hamilton Harbour had the highest concentrations of polychlorinated biphenyls and organochlorine insecticides (ppb) and contained very high concentrations of polynuclear aromatic hydrocarbons (ppm); although the levels of these compounds varied widely with sampling location in the harbor. A sediment extract from Hamilton Harbour was mutagenic in the Ames bacterial assay, both with and without microsomal activation. High levels of aromatic DNA adducts were induced in cultured mouse C3H1OT1/2 cells after in vitro exposure to Hamilton Harbour sediment extract. In two separate carcinogenicity experiments involving a sac fry microinjection assay with rainbow trout (Oncorhynchus mykiss), Hamilton Harbour sediment extract induced hepatocellular carcinomas in fish. No hepatic neoplasms were observed in fish that had been treated with sediment extract from Oakville Creek, or with extract from a reference sediment. The significance of these results is discussed in relation to the distribution of neoplasms in feral fish within western Lake Ontario.

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INTRODUCTION

Chemical contaminants have been implicated in the etiology of tumors in several fish populations inhabiting freshwater and estuarine ecosystems receiving industrial and urban pollution (Couch and Harshbarger, 1985; Mix, 1986). Neoplasms with a putative chemical etiology include epidermal papillomas and dermal carcinomas (Black, 1983; Baumann et al., 1987; Hayes et al., this volume), and liver tumors arising from hepatocyte or biliary tissue (Smith et al., 1979; Malins et al., 1984; Murchelano and Wolke, 1985; Baumann et al., 1987).

In western Lake Ontario, high prevalences of epidermal papillomas and hepatic neoplasms have been reported in white suckers (*Catostomus commersoni*) inhabiting areas receiving industrial and urban pollution (Sonstegard, 1977; Smith and Zajdlick, 1987; Smith et al., in press; Hayes et al., this volume). In addition, high prevalences of epidermal papillomas and dermal carcinomas have been noted in brown bullheads (*Ictalurus nebulosus*) inhabiting the industrialized Hamilton Harbour region of western Lake Ontario (Smith et al., in press; Hayes et al., this volume).

The primary evidence supporting an association between these neoplasms and exposure to chemical contaminants has come from epidemiological data on tumor prevalence in fish from "polluted" sites in western Lake Ontario in comparison to fish from "reference" sites receiving little or no industrial and urban pollution. The data reported in this study represents an experimental approach to establishing an association between chemical contamination and carcinogenic potential in fish.

We report the concentrations of selected organic "priority pollutants", including, polychlorinated biphenyls (PCBs), organochlorine insecticides, and polycyclic aromatic hydrocarbons (PAHs), in extracts from sediment samples collected from Hamilton Harbour and Oakville Creek in western Lake Ontario, as well as from sediments at reference sites. These extracts were tested for *in vitro* mutagenic activity with the *Salmonella*/mammalian microsome mutagenicity assay (Ames test), and were tested for the ability to induce aromatic DNA adducts in an *in vivo* assay with mammalian C3H10T1/2 cells. Extracts were also tested for carcinogenic activity in fish with a sac-fry microinjection assay with rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*).

METHODS AND MATERIALS

Study Areas

Sediment samples were collected from five sites in the southeastern basin of Hamilton Harbour and from the mouth of Oakville Creek (also known as Sixteen Mile Creek) in western Lake Ontario (Figure 1). Sediments were also collected from control sites at McLennan's Creek in South Bay, which is on the southeastern side of Manitoulin Island in Georgian Bay, Lake Huron, and at Fawn Lake, which is located in the Muskoka region of central Ontario, Canada (Figure 1, inset). Figure 2 shows the specific locations of the five sediment samples collected in Hamilton Harbour.

Hamilton Harbour is an isolated embayment surrounded by the communities of Hamilton and Burlington, which receives industrial effluents, treated municipal sewage, and discharges from Great Lakes shipping (Ontario Ministry of the Environment, 1974). Major

Figure 1: The location of Hamilton Harbour and Oakville Creek in relation to western Lake Ontario, and the location of the reference sites at Fawn Lake and South Bay (inset).

development of the steel industry, including construction of a coking plant, has occurred along the southeastern shore of the harbor. The harbor contains resident and transient populations of several fish species, including white suckers and brown bullheads. However, because of severe oxygen depletion in the hypolimnion, fish populations are largely confined to the northern and western margins of the harbor.

Oakville Creek is located approximately 20 km east of Hamilton Harbour, and much of its length is within the limits of the city of Oakville. The creek flows through rural, residential, and light industrial areas, and receives discharges from a storm sewer system.

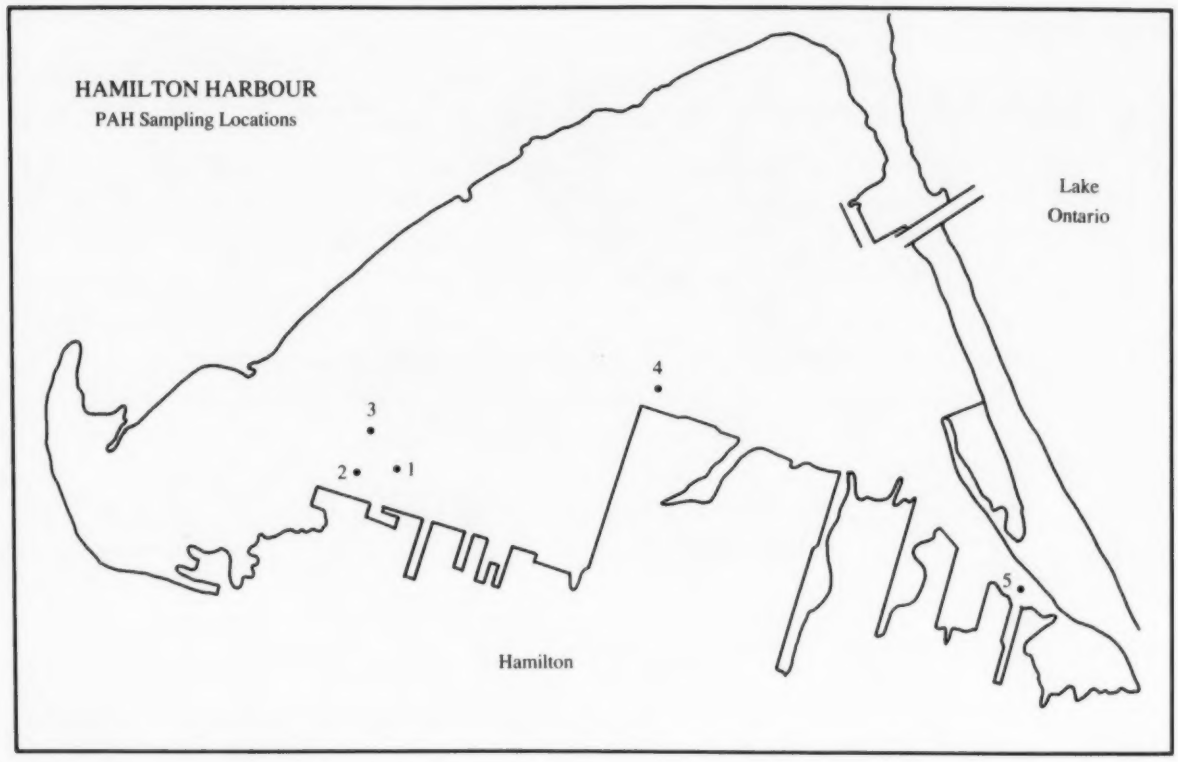

Figure 2: Hamilton Harbour, showing the location of the 5 sampling sites for the survey of PAH concentrations in sediments.

There are active and inactive sanitary landfill sites in close proximity to the creek, and there is a small marina at the creek mouth. Each spring, a spawning population of white suckers moves up the creek from Lake Ontario.

The reference sites are located in relatively remote areas of central Ontario. There are no industrial developments near either area.

Sediment Extract Preparation and Analysis

Approximately 2 kg of sediment were collected from each site with an Ekman dredge. Samples collected from station 1 in Hamilton Harbour, from Oakville Creek, and from South Bay were extracted as described by Metcalfe et al. (1988a). Briefly, samples were polytron homogenized with acetone:hexane (1:1 by volume), and the extract was allowed to separate, and dried by passing through a column of sodium sulfate. The final extract in acetone was adjusted to a volume of 12 mL (Oakville Creek, South Bay) or 24 mL (Hamilton Harbour, station 1). Subsamples (100 μL) of these sediment extracts were cleaned up by silica gel column chromatography as described by Metcalfe et al. (1988a). Samples were analyzed for PCBs and organochlorine insecticides by high resolution gas chromatography (HRGC) on a Varian 4700 GC, with dual 30 m capillary columns (DB-5, DB-1) and electron capture detectors. Samples were analyzed for PAHs by gas

chromatography-mass spectrometry (HRGC/HRMS) in selected ion mode with a Finnigan 4510 GC/MS and INCOS data system.

Sediment samples collected from stations 2 to 5 in Hamilton Harbour and from Fawn Lake were extracted and prepared for gas chromatographic analysis according to method "A" of the Ontario Ministry of the Environment protocol for analysis of organic contaminants in sediments (OME, 1983). Briefly, sediments were extracted with acetone by sonification, back-extracted into methylene chloride, and dried by passing through sodium sulfate. Extracts were cleaned up by gel permeation chromatography as described by Macdonald et al. (in press). Samples were analyzed for PAHs by HRGC with an HP 5890 GC, using dual 30 m columns (DB-1701, SPB-5) and flame ionization detectors.

The sediment extracts were stored in acetone in sealed glass ampoules at 4°C. Sediment extracts were prepared for mutagenicity and carcinogenicity tests and for assays of DNA adduct formation by taking aliquots of extracts in acetone and adding dimethylsulfoxide (DMSO). Acetone was evaporated off under nitrogen flow, and extracts were further diluted with DMSO to the desired test concentration.

Mutagenicity Assay

Sediment extracts from Hamilton Harbour (Station 1), Oakville Creek, and South Bay were diluted with DMSO to a range of concentrations equivalent to 0.3 to 20 g of sediment per mL of DMSO (i.e. 0.3 to 20 g equivalents). The dilutions used in the mutagenicity assay were determined by preliminary toxicity tests with the assay system. The *Salmonella*/mammalian microsome mutagenicity assay was conducted as described by Maron and Ames (1983) with strains TA98 and TA100, both with and without addition of microsomal solution (S-9) for exogenous enzyme activation. The S-9 solution was prepared from the livers of rats which had been treated with Aroclor 1254, and was purchased from Inter-Medico Ltd. of Willowdale, Ontario. The number of colonies in the test plates (revertant colonies) were counted in three replicate plates. A range of four doses were tested for each treatment.

In Vitro Assay for DNA Adducts

Extracts from Hamilton Harbour (Stations 2, 4, and 5) and Fawn Lake were diluted with DMSO to a concentration equivalent to 1 g of sediment per mL of DMSO. The C3H10T1/2 cells were treated *in vitro* for 3 hours with 1% DMSO or 1% DMSO extract in tissue culture medium, and then were harvested. Levels of DNA adducts in the cultured cells were determined by the ^{32}P-postlabeling method described by Dunn and San (1988). Briefly, DNA was extracted from cells and hydrolyzed to mononucleotides and their adducts. Adducted nucleotides were then purified by nuclease P1 destruction of the normal nucleotides, and labelled with ^{32}P. Adducts were then separated in two dimensions by thin-layer chromatography, and detected by autoradiography. Individual adducts were detected as dark spots on the X-ray film.

Rainbow Trout Carcinogenicity Assay

Rainbow trout of the Kamloops strain were microinjected with sediment extracts when they reached the "sac fry" stage of development, as described by Metcalfe et al. (1988b).

Figure 3: Narcotized sac-fry of rainbow trout on filter paper showing the microinjection apparatus.

During microinjection, sac fry were anesthetized by immersion in water saturated with carbon dioxide and were placed on a piece of dry filter paper, where they were immobilized by adhesion to the filter paper (Figure 3). Sac fry were injected with a microlitre syringe (25 μL capacity) equipped with a 31 gauge syringe needle. The syringe was fitted with a push-button repeating dispenser, so that solutions were dispensed in 0.5 μL volumes. Sac fry were injected by inserting the syringe needle into the yolk in an anterioposterior direction (Figure 4), and pressing the dispenser button. After all the sac fry on the filter paper were injected, the paper was placed in aerated water and fry were shaken off into a recovery vessel. After all sac fry had resumed normal activity in the recovery vessel, they were placed in raceways for rearing.

Fish were placed in raceways at 12°C, and raised through "swim-up" (start of exogenous feeding) to a size of approximately 10 g. Fish were then transferred to 500 L circular tanks and raised at water temperatures of between 10°C and 15°C to a weight of approximately 250 g, at age 12 mo. The density of fish in the circular tanks was kept constant at approximately 300 fish per tank. The trout were fed commercial trout diets *ad libitum*.

When fish were 12 mo old, they were killed by an overdose of tricaine methanesulfonate anesthetic (MS-222). The trout were examined externally and internally for grossly visible neoplasms, and their livers were removed and preserved in 10% formalin or Bouin's fixative for histological examination.

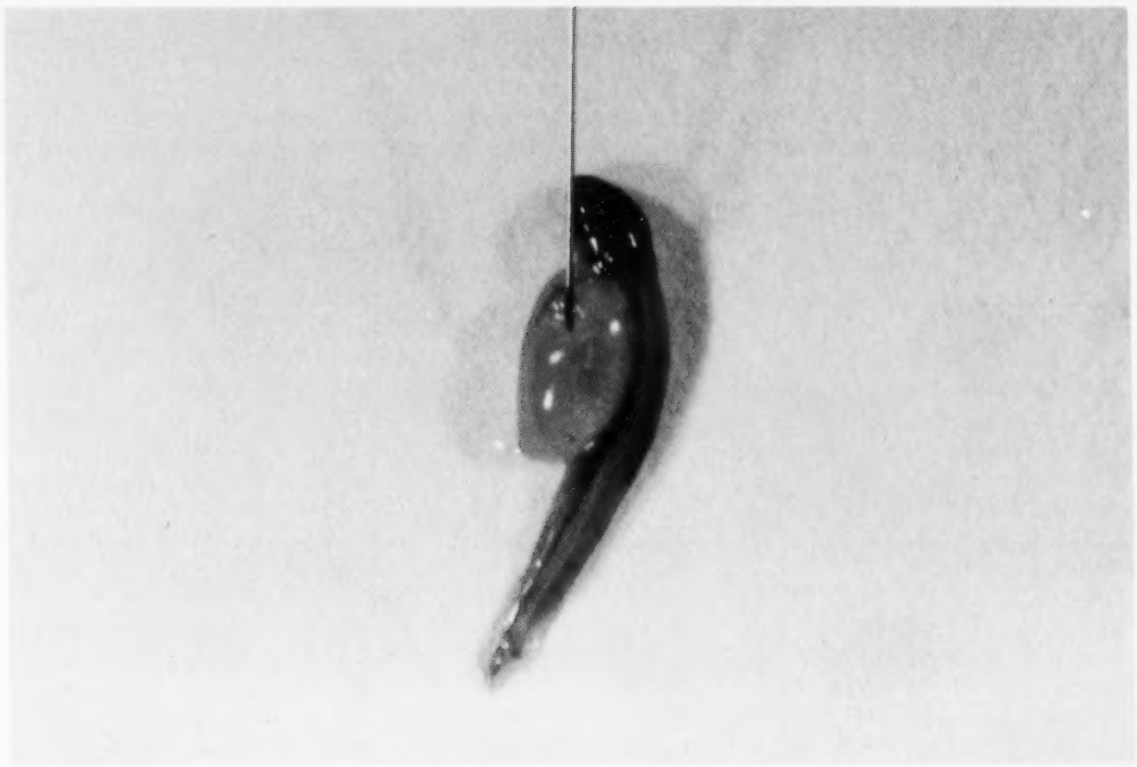

Figure 4: A narcotized sac-fry showing the position of the needle at microinjection.

In these experiments, rainbow trout were injected at the sac fry stage with DMSO (control), with 13 ng and 25 ng per embryo of AFB_1 in DMSO (positive control), and with sediment extracts at various doses between 0.003 and 0.05 g equivalents. Two separate carcinogenicity experiments were conducted with sediment extracts. In experiment I, fish were treated with two doses of extract from Oakville Creek (0.05 and 0.025 g equivalents), and one dose each of extract from Hamilton Harbour (Station 1, 0.006 g equivalents) and South Bay (0.05 g equivalents). In experiment II, fish were treated with three doses from each of the extracts prepared from sediments in Hamilton Harbour (Station 1), Oakville Creek, and South Bay.

Histopathology

Rainbow trout livers that had been dissected into 4 or 5 pieces at necropsy were embedded in paraffin, sectioned, and stained with hematoxylin and eosin in preparation for microscopic examination. Livers in which lesions had been observed grossly were extensively sectioned for histological confirmation of visual observations. All other livers were surveyed by cutting one section from the proximal face of each paraffin block and examining it microscopically for evidence of neoplasia.

The histological classification of hepatic neoplasms was based upon the criteria of Hendricks et al. (1984b). Basophilic hepatocellular carcinomas were distinguished from preneoplastic or benign basophilic lesions by increased mitotic activity in the hepatocytes,

TABLE 1

Concentrations of polycyclic aromatic hydrocarbons in sediments collected from Hamilton Harbour (5 stations), the mouth of Oakville Creek, and two control sites (South Bay, Fawn Lake). ND means "not detected" at concentrations above 0.01 $\mu g\ g^{-1}$. NA means not "analyzed".

a) Hamilton Harbour Stations

Compound	Concentration ($\mu g\ g^{-1}$)				
	Stn. 1	Stn. 2	Stn. 3	Stn. 4	Stn. 5
Naphthalene	2.03	NA	NA	NA	NA
Acenaphthylene	0.33	3.76	3.90	15.00	ND
Acenaphthene	0.40	3.40	4.16	ND	ND
Fluorene	1.39	5.10	5.72	ND	ND
Phenanthrene	11.41[a]	36.95	40.80	ND	ND
Anthracene	NA	11.66	11.73	ND	ND
Fluoranthene	5.06	51.34	51.96	32.60	ND
Pyrene	4.26	36.64	37.17	ND	ND
Benzo(a)anthracene[b]	2.36	15.75	ND	ND	ND
Benzo(b)fluoranthene	3.49	14.73	ND	ND	ND
Benzo(k)fluoranthene	0.69	ND	ND	ND	ND
Benzo(a)pyrene	4.78	ND	ND	ND	ND
Indeno(1,2,3-c,d)pyrene	0.39	19.11	ND	ND	ND
Dibenzo(a,h)anthracene	0.32	ND	ND	ND	ND
Benzo(g,h,i)perylene	1.61	14.24	ND	ND	ND

[a] Total of phenanthrene and anthracene.
[b] Total of chrysene and benzo(a)anthracene.

b) Oakville Creek and Control Stations

	Oakville Creek	South Bay	Fawn Lake
Naphthalene	ND	ND	NA
Acenaphthylene	ND	ND	ND
Acenaphthene	ND	ND	ND
Fluorene	ND	ND	ND
Phenanthrene	0.04	ND	ND
Anthracene	ND	ND	ND
Fluoranthene	0.03	ND	ND
Pyrene	0.02	ND	ND
Benzo(a)anthracene	0.01	ND	ND
Benzo(b)fluoranthene	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND
Benzo(a)pyrene	ND	ND	ND
Indeno(1,2,3-c,d)pyrene	ND	ND	ND
Dibenzo(a,h)anthracene	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND

compression of surrounding tissue, and increased hepatic cord width (>2 cells wide).

The total incidence of hepatic carcinomas was determined by summing the results of visual and histological surveys. Only hepatic carcinomas, and not preneoplastic or benign lesions, were included in statistical analysis of incidence data. The frequencies of hepatocellular carcinomas in trout were compared statistically by means of chi-square contingency analysis with a continuity correction, as outlined by Armitage (1971).

RESULTS

Chemical Analysis of Sediment Extracts

The concentrations of PCBs and organochlorine pesticides were relatively low in sediments collected from Hamilton Harbour. Hexachlorobenzene and PCBs were detected in Hamilton Harbour (station 1 analyzed, only) at concentrations of 0.6 ng g^{-1} and 20.9 ng g^{-1}, respectively. Only PCBs were detected in the sediments from Oakville Creek, South Bay, and Fawn Lake, at concentrations below 5 ng g^{-1}.

The concentrations of selected PAHs in sediments from Hamilton Harbour stations 1, 2 and 3 were in the $\mu g\ g^{-1}$ range (Table 1). PAHs in samples from stations 1 and 2 included high molecular weight, multi-ringed compounds, but the only PAH compounds detected at station 3 were all of low molecular weight. It is important to note that the sample from Hamilton Harbour station 1 contained measurable quantities of benzo(a)pyrene (BaP), a carcinogenic PAH (4.78 $\mu g\ g^{-1}$), but the sample from station 2 did not. Sediments from Hamilton Harbour station 4 and Oakville Creek contained only a few low molecular weight PAHs, and sediments from Hamilton Harbour station 5 and Fawn Lake had no detectable concentrations of PAH compounds.

Mutagenicity of Sediment Extracts

The mutagenicity of all sediment extracts was relatively low (Table 2). In tests of the extract from Hamilton Harbour station 1 with the TA100 *Salmonella* strain, there was a

Table 2: Mutagenicity of extracts from Hamilton Harbour (Station 1), Oakville Creek and South Bay sediments tested with TA100 and TA98 Salmonella strains, with and without S-9 activation.

Treatment	Mean No. of Revertants (n=3) TA100		TA98	
	With S-9	Without S-9	With S-9	Without S-9
a) Hamilton Harbour (Stn. 1)				
2.5 g equivalents	202	203	61	64
1.2	211	209	52	60
0.6	194	163	49	47
0.3	188	114	39	45
Spontaneous	102	105	33	39
b) Oakville Creek				
10.0 g equivalents	128	Toxic	32	32
5.0	117	Toxic	40	42
2.5	92	15	41	37
1.2	103	47	40	38
Spontaneous	98	101	40	38
c) South Bay				
20.0 g equivalents	90	Toxic	44	32
10.0	104	30	32	38
5.0	101	32	38	32
2.5	93	57	40	38
Spontaneous	97	97	38	39

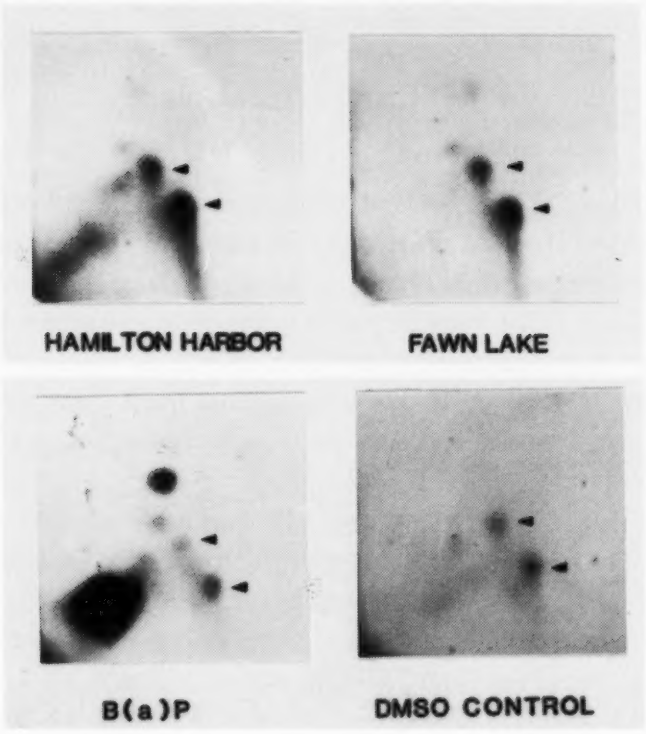

Figure 5: Autoradiograms of chromatograms showing chemical-DNA adducts formed after <u>in vitro</u> incubation of C3H10T1/2 cells with solutions of benzo(a)pyrene (BaP), DMSO control, and extracts prepared from sediments of Hamilton Harbour and Fawn Lake (reference site).

doubling of revertants above spontaneous levels and a dose-response relationship; both with and without the addition of S-9. This indicates significant mutagenic activity according to the criteria of Johnson and Hopke (1980). With strain TA98, treatment with the Hamilton Harbour extract elevated the numbers of revertants, but not to twice the spontaneous value. Doses of Hamilton Harbour extract greater than 2.5 g equivalents were toxic to the test strains.

There was no indication of mutagenic activity in tests with sediment extracts from Oakville Creek and the reference site (South Bay). In both cases, the extracts were toxic to the test strains at the highest concentrations, as indicated by numbers of revertants below spontaneous values or by total inhibition of bacterial growth (Table 2). The toxicity of extracts was particularily great in tests without S-9 activation.

DNA Adduct Analysis of Sediment Extracts

In the *in vitro* assay with C3H10T1/2 cells, treatment with extracts from Hamilton Harbour station 2 and station 4 gave autoradiography patterns consisting of a diffuse, diagonal radioactive zone (Figure 5). In comparison, the autoradiograph from the treatments with sediment extract from Hamilton Harbour station 5, Fawn Lake (reference site) and the DMSO control exhibited only two background spots (see arrows in Figure 5).

Table 3: Numbers of fish with hepatic neoplasms and total per cent of fish with hepatocellular carcinomas for rainbow trout necropsied 12 mo after injection at the sac fry stage with aflatoxin B_1 (AFB1), or with sediment extracts from Hamilton Harbour (Station 1), Oakville Creek, and South Bay.

Treatment[a]	N	Numbers of Trout with Hepatic Neoplasms			
		Visual Survey[b]	Histologic Survey[c]	Total Carcinomas[d] #	%
a) Experiment I:					
DMSO Control	15	0	0	0	0
AFB1 (13 ng)	81	4	5	9	11.1
South Bay (0.05g)	62	0	0	0	0
Oakville Ck. (0.05g)	87	0	0	0	0
Oakville Ck. (0.025g)	37	0	0	0	0
Hamilton Hbr. (0.006g)	123	8	3	11	8.9
b) Experiment II:					
DMSO Control	168	0	0	0	0
AFB1 (25 ng)	130	26	12	38	29.2
South Bay (0.05g)	131	0	0	0	0
South Bay (0.025g)	98	0	0	0	0
South Bay (0.012g)	88	0	0	0	0
Oakville Ck. (0.05g)	131	0	0	0	0
Oakville Ck. (0.025g)	98	0	0	0	0
Oakville Ck. (0.012g)	88	0	0	0	0
Hamilton Hbr. (0.012g)	148	7	5	12	8.1
Hamilton Hbr. (0.006g)	124	3	2	5	4.0
Hamilton Hbr. (0.003g)	65	2	0	2	3.1

[a] Dose of sediment extracts calculated as equivalent wet weight of sediment injected into sac fry (g equivalents).

[b] Numbers of fish with hepatic neoplasms observed grossly. All gross observations confirmed by histological examination as hepatocarcinomas.

[c] Numbers of additional fish with hepatocarcinomas detected by histological survey.

[d] Total number of fish with hepatocarcinomas detected by visual survey plus histological survey.

Treatment with BaP (10^{-6} M) induced a different autoradiography pattern, with two discreet radioactive zones in the lower left and upper central part of the chromatography plate.

Carcinogenicity of Sediment Extracts

The results of carcinogenicity experiments I and II are summarized in Table 3. No grossly visible lesions, besides hepatic neoplasms, were noted externally or internally in any

of the trout examined from experiment I or experiment II. Data are presented on the number of hepatic neoplasms observed grossly, plus the number of malignant hepatic lesions (hepatocellular carcinomas) observed in the histological survey. The neoplasms observed grossly were all confirmed histologically as hepatocellular carcinomas. A previous study on the efficiency of this survey method for enumerating tumors (Metcalfe and Sonstegard, 1985), indicated that visual plus histological surveys detected over 90% of the trout with hepatocarcinomas, but the visual survey alone only detected 73%. Not included in Table 3 are the numbers of preneoplastic or benign basophilic lesions observed histologically in the liver tissue.

In experiment I (Table 3a), there were gross hepatic neoplasms seen in 8 of the 123 trout which were necropsied 12 mo following microinjection with 0.006 g equivalents of sediment extract from Hamilton Harbour (station 1). All of these neoplasms, which were relatively small (1-3 mm), whitish, nodular areas, were confirmed histologically as hepatocellular carcinomas. An additional three fish from this treatment had hepatocellular carcinomas which were not noted in the visual survey. The total number of hepatic carcinomas noted in the Hamilton Harbour treatment represented approximately 9% of the necropsied trout. No hepatic neoplasms were noted in trout which had been microinjected with South Bay (reference site) or Oakville Creek sediment extracts, or among control trout which had been microinjected with DMSO. Hepatocellular carcinomas were observed in rainbow trout exposed to 13 ng of the potent hepatocarcinogen, aflatoxin B_1 (positive control).

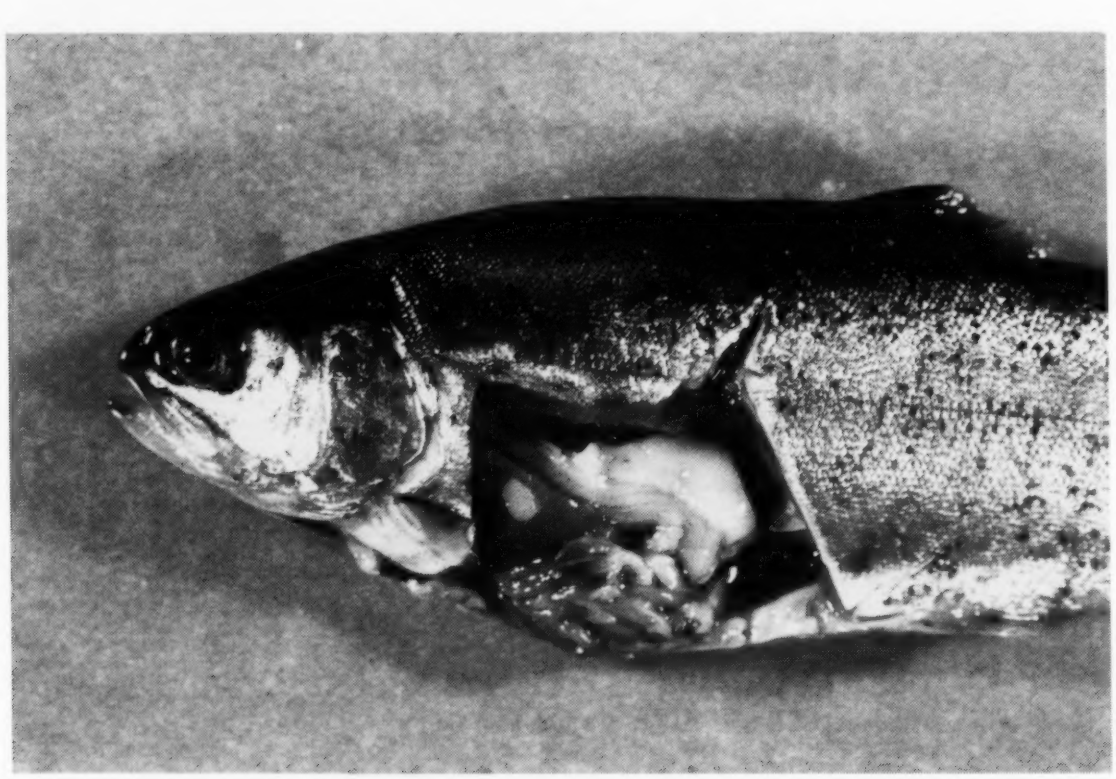

Figure 6: A neoplasm grossly visible on the surface of the liver of a juvenile rainbow trout after microinjection of carcinogen in the sac-fry stage.

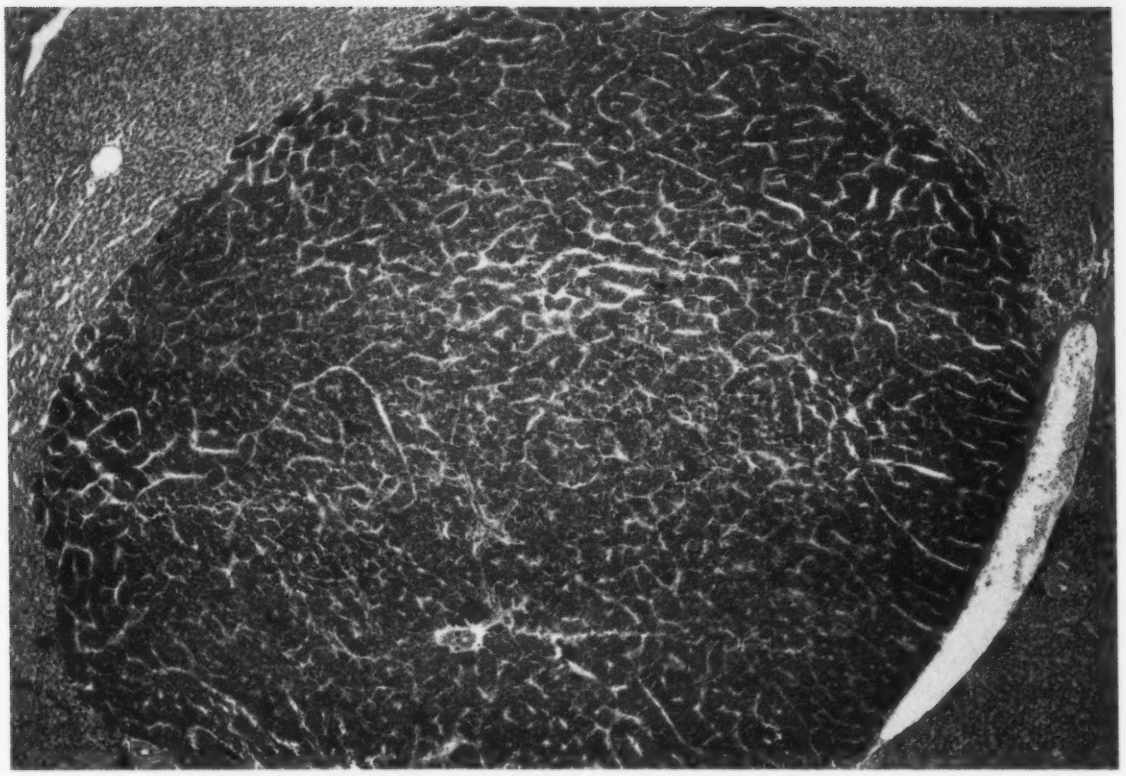

Figure 7: A photomicrograph of a trabeculate hepatocarcinoma induced after microinjection with sediment extract from Hamilton Harbour (x 40).

In experiment II (Table 3b), there were gross hepatic neoplasms observed in trout treated with all three doses of Hamilton Harbour sediment extract, with the greatest number of gross neoplasms (7 of 148) observed among trout receiving the highest dose (0.0125 g equivalents). These lesions, which were confirmed histologically as hepatocellular carcinomas, were usually small (1-3 mm), but in two cases were approximately 10 mm in diameter (Figure 6). Several additional fish from the Hamilton Harbour treatments had histologically visible hepatocellular carcinomas, which were not observed in the initial visual survey (Table 3b). When the results of the visual and histological surveys were combined, the percent incidence noted among Hamilton Harbour treatments in experiment II showed a three point dose-response. However, the difference between the percent carcinoma incidence at the two lowest doses was not statistically significant. Again, no hepatic neoplasms of any kind were noted in the treatments with South Bay (reference site) or Oakville Creek sediment extracts, or in the DMSO controls. Fish treated with 25 ng of AFB_1 had a high incidence of hepatocellular carcinomas (Table 3b).

Histologically, hepatocellular carcinomas noted in Hamilton Harbour treatments usually consisted of nodular areas of strongly basophilic hepatocytes arranged in a trabecular pattern (Figure 7). There was compression of surrounding normal hepatocytes as a result of rapid growth, and a few mitotic figures. With some of the larger trabeculate carcinomas, there were areas of fibrosis and/or hemorrhaging in the center of the lesion. The neoplasms induced by Hamilton Harbour sediment extract closely resembled the hepatocellular carcinomas induced by aflatoxin B_1. There was one atypical hepatocellular carcinoma

(described in Metcalfe et al., 1988a) observed in a Hamilton Harbour treatment (experiment II, 0.003 g equivalents), in which neoplastic hepatocytes were arranged in dense rosette formations.

DISCUSSION

In two separate experiments, extracts from sediment collected in Hamilton Harbour (Station 1) induced malignant hepatic tumors in rainbow trout. In general, the carcinomas induced by sediment extracts resembled closely the hepatocellular neoplasms induced in rainbow trout by known carcinogens, such as aflatoxin B_1 and dimethylnitrosamine (Hendricks et al., 1984a), BaP (Black et al., 1985; Hendricks et al., 1985), and 7,12-dimethylbenzanthracene (Metcalfe and Sonstegard, 1984).

The hepatocellular carcinomas induced in rainbow trout by chemical carcinogens are considered true malignancies by virtue of their potential for metastasis, and their transplantability (Hendricks et al., 1984b). However, the malignant nature of the lesion may not be apparent until late in the life of the fish, and the fish may die from indirect effects of the tumor, well before the primary malignancy or metastatic lesions become life-threatening.

In addition to showing carcinogenic activity, Hamilton Harbour sediment extract was mutagenic in the Ames test, induced DNA adducts in mammalian cells in an *in vitro* assay, and contained high concentrations of PAH compounds. Levels of PAHs in sediments were similar to those reported by Poulton (1987) for Hamilton Harbour, and are similar to PAH concentrations in other highly polluted sediments (Baumann et al., 1987; Black, 1983; Malins et al., 1984). Many of the PAH compounds, such as BaP, dibenz(a,h)anthracene, and benzanthracenes, are recognized mutagens/carcinogens, while other compounds are known tumor promotors (e.g. chrysene). The analytical survey at five sites in the southeastern part of Hamilton Harbour indicated that the distribution of PAH compounds in sediment is not uniform, with both the concentration and PAH composition varying over a relatively small area.

It cannot be inferred from these limited data that PAH compounds were responsible for all of the genotoxic and carcinogenic activity in the sediment extract. Ames test data showing that the Hamilton Harbour extract was mutagenic even without S-9 activation indicate that mutagenicity was at least partly due to the presence of mutagens that do not require addition of exogenous metabolic enzymes. PAH compounds are all indirect-acting mutagens/carcinogens which require addition of exogenous microsomal enzymes (S-9) to Ames plates for metabolic activation (McCann et al., 1975). It is possible that compounds such as nitro polycyclic aromatic compounds (e.g. nitropyrene), which do not require S-9 activation in the Ames assay (Beland et al, 1985), may be responsible for all or part of the mutagenic activity in the Hamilton Harbour extract.

In the DNA adduct assay, the C3H10T1/2 cell line is capable of metabolizing PAH compounds to their reactive intermediates without the addition of exogenous enzymes. Thus, adduct formation during treatment with Hamilton Harbour extracts could have been due to either indirect or direct-acting compounds. The diffuse, diagonal pattern of the autoradiograms from the Hamilton Harbour treatments are very similar to the patterns seen in fish collected from the Buffalo River and Detroit River (Dunn et al., 1987; Maccubbin et al., this volume), and fish collected from Puget Sound (Stein et al., this volume); areas

also contaminated with PAH compounds. The autoradiogram pattern induced by sediment containing a complex mixture of aromatic compounds (i.e. Hamilton Harbour sediment) is different from the pattern induced by exposure to the single PAH compounds, BaP. DNA adducts are the initial biochemical lesions thought to be involved in mutagenesis and carcinogenesis by aromatic compounds. Thus, it is likely that aromatic compounds present in Hamilton Harbour sediment are responsible for the mutagenic and carcinogenic activities observed in the Ames and trout microinjection assays, respectively.

In experiment I of the trout embryo microinjection assay, the Hamilton Harbour sediment extract induced hepatic carcinomas in 8.9% of the necropsied trout at a dose of 0.006 g equivalents per embryo, while in experiment II, this same dose only induced hepatic carcinomas in 5.0% of the necropsied fish. This reduction in carcinogenic activity between the two experiments may indicate some degradation of the active components of the mixture in the intervening 5 mo, even though the extracts were stored at 4°C in the dark.

These data show that sediment-bound contaminants in Hamilton Harbour have the potential for inducing malignant tumors in exposed fish species. In several areas of western Lake Ontario, including Hamilton Harbour, the prevalence of lip and body papillomas among bottom-dwelling white suckers is elevated (Sonstegard, 1977; Smith and Ferguson, 1985; Smith et al., in press). Lip and body papillomas and epidermal carcinomas are also present in brown bullheads in Hamilton Harbour (Smith et al., in press; Hayes et al., this volume). Perhaps more indicative of chemically-induced carcinogenesis are the high prevalences of hepatic tumors in white suckers populations within several areas of western Lake Ontario, including both Hamilton Harbour and Oakville Creek (Smith and Zajdlick, 1987; Hayes et al., this volume).

It is not surprising, therefore, that Hamilton Harbour sediments gave a positive response in these assay systems. However, it is unexpected that sediment extract from the mouth of Oakville Creek had no mutagenic or carcinogenic activity, and contained very low levels of organic contaminants. However, the white sucker is a relatively mobile species, and tumor-bearing individuals sampled from Oakville Creek could have been exposed to chemical carcinogens from several areas in western Lake Ontario before being captured.

These data indicate that there is the potential for the development of tumors in feral fish from western Lake Ontario as a result of exposure to chemical carcinogens in Hamilton Harbour. However, it is not clear whether fish are ever in close proximity to the areas of heaviest contamination in Hamilton Harbour. The contaminated sediments occupy a small area in the southeastern part of the harbor, which may be largely inaccessable to fish because of anoxic conditions (V. Cairns, personal communication). In addition, there is no direct evidence that contaminants bound to bottom sediments are able to induce tumors in populations of fish; although, bottom-dwelling fish are the species most frequently afflicted with epithelial and hepatic tumors in contaminated regions (Couch and Harshbarger, 1985). More work is required to determine whether sediment-bound contaminants can be taken up into fish in carcinogenic doses as a result of direct contact with sediment, or by absorption through the gills, or through consumption of contaminanted benthic organisms.

ACKNOWLEDGEMENTS

This study was supported by a strategic grant from the Natural Sciences and Engineering Council of Canada to C.D.M, and by an operating grant from NSERC to B.P.D. The

authors wish to thank Michelle McWhirter, Bill Gibson, Marsha Ferguson, Jan Campfens, and Nakos Kotsanis for technical assistance on the project. Histopathology was confirmed by Richard Moccia, Aquatic Pathology Services, Guelph, Ontario.

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The Science of the Total Environment, 94 (1990) 143–153
Elsevier

A POSSIBLE CORRELATION BETWEEN ENVIRONMENTAL CHEMICALS AND PIGMENT CELL NEOPLASIA IN FISH

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ABSTRACT

A croaker (Nibea mitsukurii) has a high incidence of the skin melanoma, chromatophoroma, in a Pacific coastal area in Japan. A sea catfish (Plotosus anguillaris) bearing skin melanosis is also found in the same area. For elucidation of a correlation between these pigment cell neoplasms of the skin and environmental contaminants, an epidemiological survey was conducted to determine the distribution and prevalence of tumor-bearing fish. Based upon observations of a high prevalence of skin neoplasms near the discharge point for kraft pulp mills, experiments were conducted to determine the neoplastic induction efficiency of the effluent on the croaker and sea catfish species. Isolation and identification of mutagens in effluent extracts were carried out using the Ames test, followed by mass spectral analysis of mutagenic fractions. The effluent induced a chromatophoroma on one croaker of the 100 tested, and it induced pigment cell hyperplasia on 70 to 100% of the sea catfish. These skin neoplasms were grossly similar to those observed in the field. Five chloroacetones were identified from the Ames-positive fractions of the effluent, and tetrachlorocyclopentene-1,3-dione and two α-dicarbonyl compounds were also detected as mutagens. The above experiments indicate that the mutagenic contaminants found in kraft mill effluent may play an important role in the induction of skin neoplastic disease in fish.

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INTRODUCTION

Since the end of the eighteenth century, 300 fish species bearing neoplastic diseases have been reported (Wellings, 1969). The types of neoplastic diseases in fish are very similar to those observed in mammals. Epizootiological studies suggest that the occurrence of these fish diseases is correlated with various environmental factors, such as bacterial and parasitic infections, and exposure to chemical contaminants (Snieszko, 1975; Sindermann, 1979). However, until recently, there were few reports which demonstrated a direct correlation between environmental chemicals and fish tumors. In 1941, skin carcinomas were reported in brown bullheads (*Ictalurus nebulosus*) inhabiting a polluted river (Luckē and Schlumberger, 1941). A clear correlation between fish tumors and exposure to chemical carcinogens was demonstrated in studies of hepatomas in rainbow trout in 1960. Aflatoxins produced in fish meal induced hepatic tumors in domestic trout (Halver and Mitchell, 1967).

Pigment cell neoplasms are a common disease in fish, as they have been reported in 50 fish species. In 1973, it was observed that the croaker (*Nibea mitsukurii*), inhabiting the coastal areas of Japan, had a high incidence of the skin melanoma, chromatophoroma. In the period from 1973 to 1981, 70 stations along the coast of Japan were surveyed, and the results of epizootiological and epidemiological studies of this fish tumor were reported (Kimura et al., 1984). An elevated prevalence of the chromatophoroma was unique to a particular sampling station located on the Kii peninsula of Japan.

The coast of the Kii peninsula is generally free from urban waste discharges, and is sparsely populated. However, wastewater from a large kraft pulp mill is discharged into the coastal area near the sampling station at which a high prevalence of the chromatophoroma was detected in the croaker population. Recently, several mutagenic compounds have been identified in the effluents of a kraft pulp mill, including neoabietic acid, tetrachloropropene, pentachloropropene, 1,3-dichloroacetone, and 2- chloropropenal (Kringstad et al., 1981; Holbom et al., 1981; Nestman et al., 1985). It is possible that these genotoxic chemicals in kraft effluents are responsible for the induction of skin carcinomas in croakers.

In this paper, we report recent epidemiological data on the pigment cell neoplasms in the croaker, and also in the sea catfish (*Plotosus anguillaris*), with an emphasis on the possible role of environmental contaminants in the induction of these fish diseases. We report the results of experiments directed at inducing skin neoplasms on croakers and sea catfish by exposure to kraft mill effluents. The mutagenic activity of extracts prepared from kraft pulp mill effluent are reported, and gas chromatography/mass spectrometry (GC-MS) analyses are used to identify mutagenic compounds extracted from kraft mill effluents. Several of the identified compounds are tested for mutagenicity in the *Salmonella* mutagenicity assay (Ames test).

METHODS AND MATERIALS

Epidemiological Studies

Fish were captured with the help of sport-fishermen by rod and line, and with casting

nets. Fish were examined grossly for the presence of tumorous lesions, and any diagnosis of neoplasia was confirmed by histological examination (H&E staining) under a microscope.

Induction of Neoplastic Disease

Using techniques for hatching and rearing developed by Taniguchi (1981), croaker were reared in tanks to 50 days of age. Croaker were then exposed in sea water to 10% dilutions of two types of kraft pulp mill effluent (Effluents D and E). The effluent samples were taken at two sites in the mill, before (D) and after (E) final treatment preceeding discharge into the sea. The fish were exposed twice a month, for 24 h periods, to a total of 7 exposures. Sea catfish caught in the Kii peninsula region were also exposed in sea water to 10% dilutions of the two kraft pulp mill effluents, as described for the induction experiments with croaker.

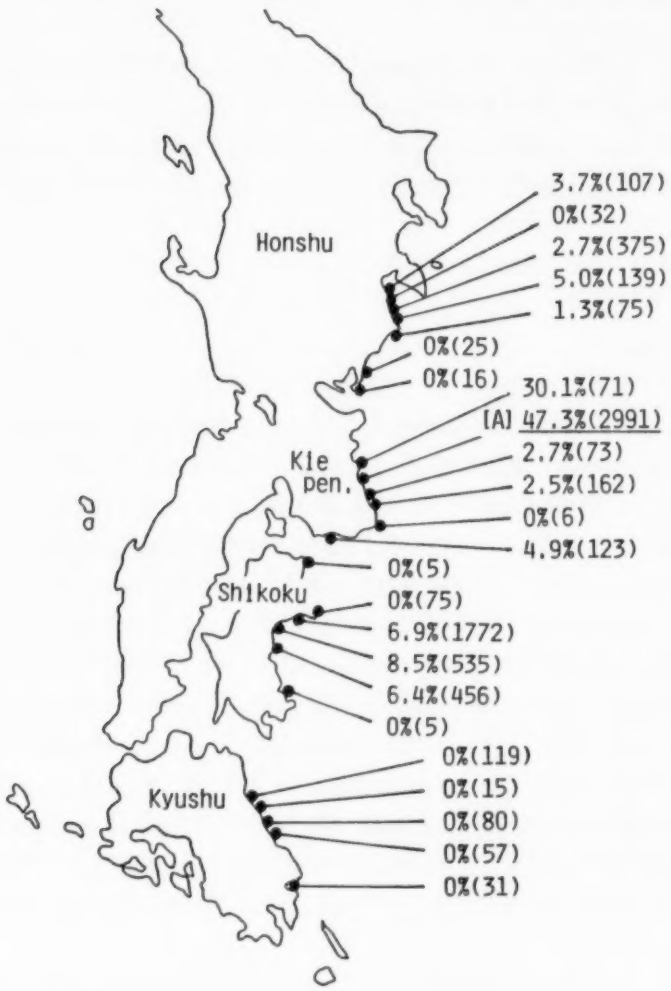

Figure 1: Geographic distribution of mean prevalence of chromatophoromas in croaker (Nibea mitsukurii) during surveys along the coast of Japan from 1973 to 1981. The numbers in parentheses are the number of fish observed.

Isolation and Identification of Mutagens from Environmental Samples

At point A on the Kii peninsula (Figure 1), samples were collected of coastal seawater (A), domestic wastewater (B), river water (C), kraft pulp mill effluents before and after final treatment (D, E), and chlorination stage effluent from the pulp mill (C-stage). Samples of 20-40 L volume were passed through an Amberlite XAD-2 resin column (2 cm x 30 cm). The resin was then removed from the column and the adsorbate extracted from the resin in a Soxhlet apparatus, first with diethyl ether, and then with methanol. After evaporation of the solvent, the residue was dissolved in dimethylsulfoxide (DMSO) in preparation for mutagenicity testing.

Ames-positive mutagenic extracts were further purified by gel filtration chromatography using a Sephadex LH-20 column (2.2 cm x 30 cm). The effluent from this column was then separated into 5 subfractions by high performance liquid chromatography (HPLC). The most mutagenic of the HPLC subfractions were analyzed by high resolution gas chromatography/mass spectrometry (HRGC-MS), according to the methods described by Kimura et al. (1989).

The *Salmonella*/mammalian microsome mediated mutagenicity assay (Ames test) was conducted as described by Ames et al. (1975), using S. *typhimurium* strains TA100 and TA98. Ames tests were conducted both with and without exogenous activation by microsomal enzyme solutions (S-9), prepared from the livers of Aroclor 1254-induced rats.

RESULTS

Epidemiological Studies

The croaker, N. *mitsukurii* (Sciaenidae, Percina, Percide, Teleostei) called "nibe" in Japanese, is a common fish inhabiting coastal waters along the Pacific coast from the north-eastern to the south-western portions of the island of Japan. The skin melanomas, described as chromatophoromas, observed on croakers were grossly visible as black, grey, white, or mixed-color lesions on the skin. Small tumors were approximately 4 mm in diameter, and large tumors were often more than 4 cm in diameter, and invaded adjacent muscle layers. The prevalence rate was correlated to the size of the fish caught; as no tumor was observed in fish less than 209 mm in total body length (younger than about 1.5 years old). As illustrated in Figure 1, the prevalence of chromatophoromas in croaker during surveys from 1973-1981 was highest at station A on the Kii peninsula (47.3%), and the tumor prevalence at other stations was between 0 and 8.5%. The variation in annual tumor prevalence in croaker at station A from 1973 to 1987 is shown in Figure 2. The average prevalence, which was 47 per cent in 1973 to 1981, decreased to 20 per cent in 1982 to 1987.

The sea catfish, P. *anguillaris* (Plotosidae, Silurina, Cyprinida, Teleostei), which is called "gonzui" in Japanese, has a high prevalence of skin melanosis, called pigment cell hyperplasia, at station A. This lesion is observed as distinctive black or grey nerve-like spots on the skin. The prevalence of this lesion on sea catfish at 11 survey stations on the Japanese coast from 1979 to 1980 was 0 to 13.5%. At station A, the 13.5% prevalence of pigment cell hyperplasia was significantly higher than the prevalence rate at other stations (0-4.8%). At station A, other tumors and related disorders were observed in various fish

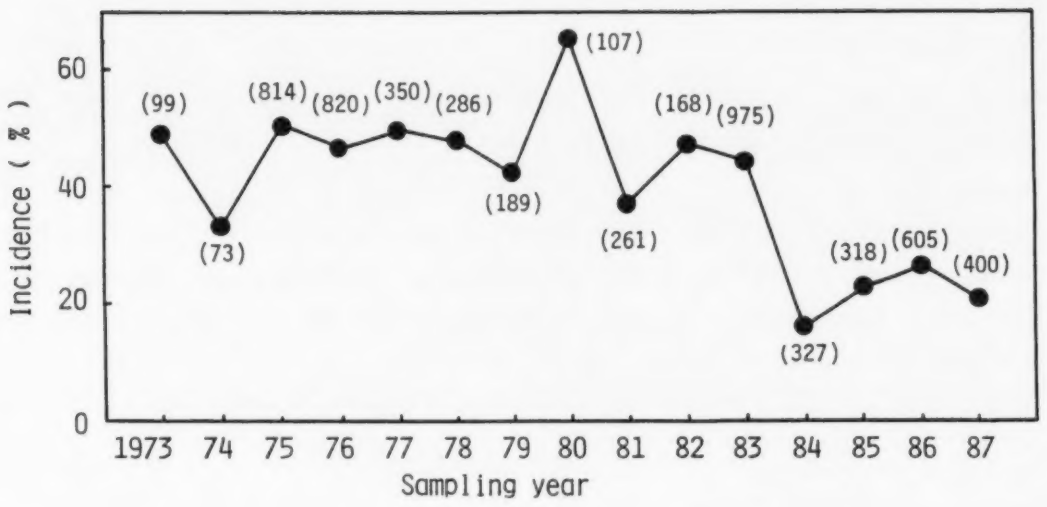

Figure 2: Variations in the prevalence of chromatophoromas in croaker (Nibea mitsukurii) during surveys at Station A from 1973 to 1987.

species. Hepatomas, gastric adenomas, and neurilemmomas were observed in the tongue sole (*Areliscus joyneri*), in the black porgy (*Acanthopagrus schlegeli*), in the Japanese sea perch (*Lateolobrax japonicus*) and in the jack (*Trachurus japonicus*). However, the data for these fish tumors were not subjected to epidemiological analysis because of the small number of samples.

Induction of Skin Neoplasms

When the croaker (100 fish) at 50 days of age were exposed to sea water containing 10% kraft pulp mill effluent E, a chromatophoroma was induced on the dorsal fin of one fish after 13 mo. In the case of sea catfish exposed to effluents D and E, skin pigment cell hyperplasia was induced in 70 to 100% of the fish after 13 mo, as shown in Table 1.

The skin lesions induced by exposure to effluents were histologically similar to those of feral fishes. Both induction tests suggest that environmental chemicals, especially contaminants in kraft pulp mill effluent, appear to be potent carcinogens for the induction of pigment cell neoplasms in fish.

Isolation and Identification of Mutagens

The ether or methanol extracts from samples A, B, and C did not show any significant mutagenic activity in the Ames test, with either tester strain, with or without addition of S-9. However, the ether extract of effluents D and E showed high mutagenic activity in Ames tests with S. *typhimurium* strain TA100, without S9 mix. As illustrated in Figure 3, the between-sample variability in mutagenic activity of these effluents was relatively large. Methanol extracts of these effluents showed no mutagenic activity with either strain. Ether extracts of C-stage effluents were found to be extremely mutagenic in the Ames test. The

TABLE 1

Incidence of skin pigment cell hyperplasia (melanosis) in sea catfish (*Plotosus anguillaris*) exposed to pulp mill effluents D and E.

Effluent	No. of fish examined	No. of fish with melanosis	Incidence (%)	No. of lesions per fish
D	7	7	100	3.00
E	20	14	70.0	1.55
Control	10	1	10.0	0.10

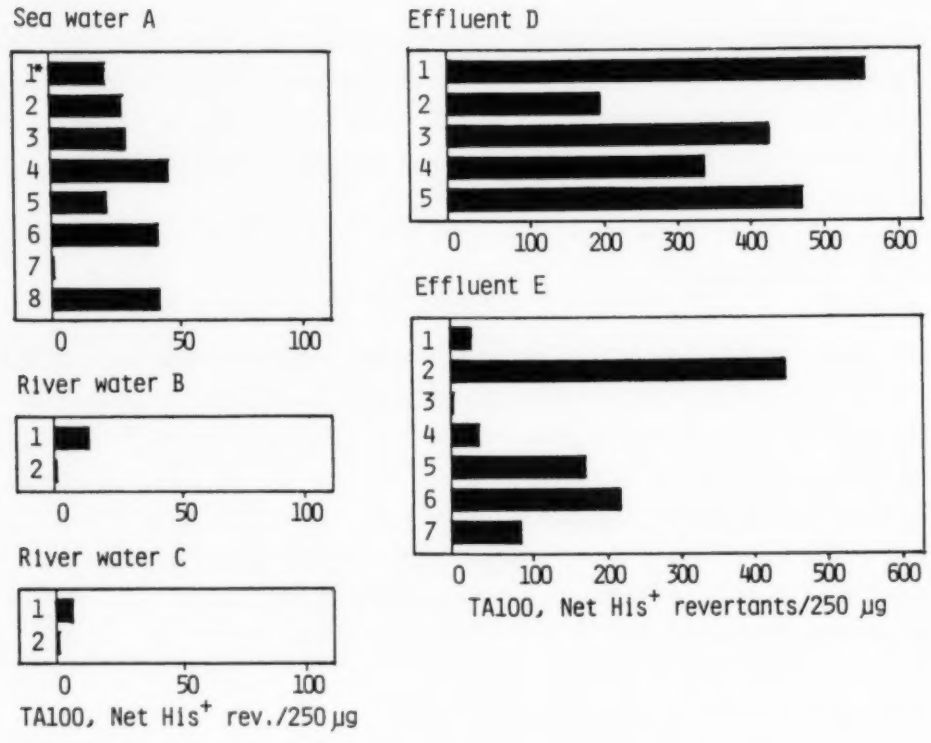

Figure 3: Mutagenic activity, as determined in the Salmonella mutagenicity assay with tester strain TA100 (without S-9), of ether extracts from water and pulp mill effluent samples. Mutagenicity is expressed in terms of net revertants per 250 µg of sample residue per plate, for between 2 and 8 samples from each site.

mean mutagenic activity of the ether extracts of the effluents, calculated as net revertants per L of original sample, were 667,000 rev L^{-1} for C-stage effluent, 4,070 rev L^{-1} for effluent D, and 580 rev L^{-1} for effluent E (Table 2).

After mutagenicity-directed subfractionation and GC-MS analysis of effluent samples D and E, several chloroacetones were identified, including 1,3-dichloroacetone,

TABLE 2

Contribution ratio of the mutagenic activity of identified compounds to the total mutagenic activity of ether extracts from D, E and C-stage effluents of a Kraft pulp mill.

Compounds	Mutagenicity (net, rev L^{-1})			Contribution ratio (%)		
	C-stage	D	E	C-stage	D	E
DiCl.acetone[a]	3,240	87	0	0.49	2.14	0
TriCl.acetone[b]	18,300	496	0	2.74	12.2	0
TetCl.acetone[c]	35,100	215	0	5.27	5.28	0
PenCl.acetone[d]	4,320	4	0	0.65	0.10	0
HexCl.acetone[e]	6	0	0	0	0	0
TCCP-dione[f]	740	0	0	0.11	0	0
methylglyoxal	1,570	140	54	0.24	3.44	9.3
diacetyl	1	0	1	0	0	0.2
Sum of identifed compounds	63,300	943	55	9.5	23.2	9.5
Total activity of ether ext.	667,000	4,070	580	(100)	(100)	(100)

[a] 1,3-dichloroacetone
[b] 1,1,3-trichloroacetone
[c] 1,1,3,3-tetrachloroacetone
[d] pentachloroacetone
[e] hexachloroacetone
[f] tetrachlorocyclopentene-1,3-dione

1,1,3-trichloroacetone, 1,1,3,3-tetrachloroacetone, and pentachloroacetone. When these compounds were tested for mutagenic activity in the Ames test, they gave a strong positive response with strain TA100, without S-9 (Figure 4). In addition to the above compounds, hexachloroacetone and tetrachlorocyclopentene-1,3-dione were detected in the C-stage effluent, and also gave a positive response in the Ames test (Figure 4).

The mutagenic activity of each effluent sample was reduced by the addition of sodium disulfite solution. This phenomenon suggests that several mutagenic carbonyl compounds are present in the effluent. On preparation of the corresponding quinoxaline derivatives of the putative carbonyl compounds by addition of o-phenylenediamine to the water samples, methylglyoxal and diacetyl were isolated and identified. These compounds are also mutagenic in the Ames assay with strain TA100 without S-9, inducing 1200 revertants per 20 μg and 20 revertants per 250 μg, respectively. The contribution of each of the identifed compounds to the total mutagenic activity of ether extracts from D, E, and C-stage effluent are summarized in Table 2. The total contribution values were 23.2, 9.5, and 9.5 %, respectively.

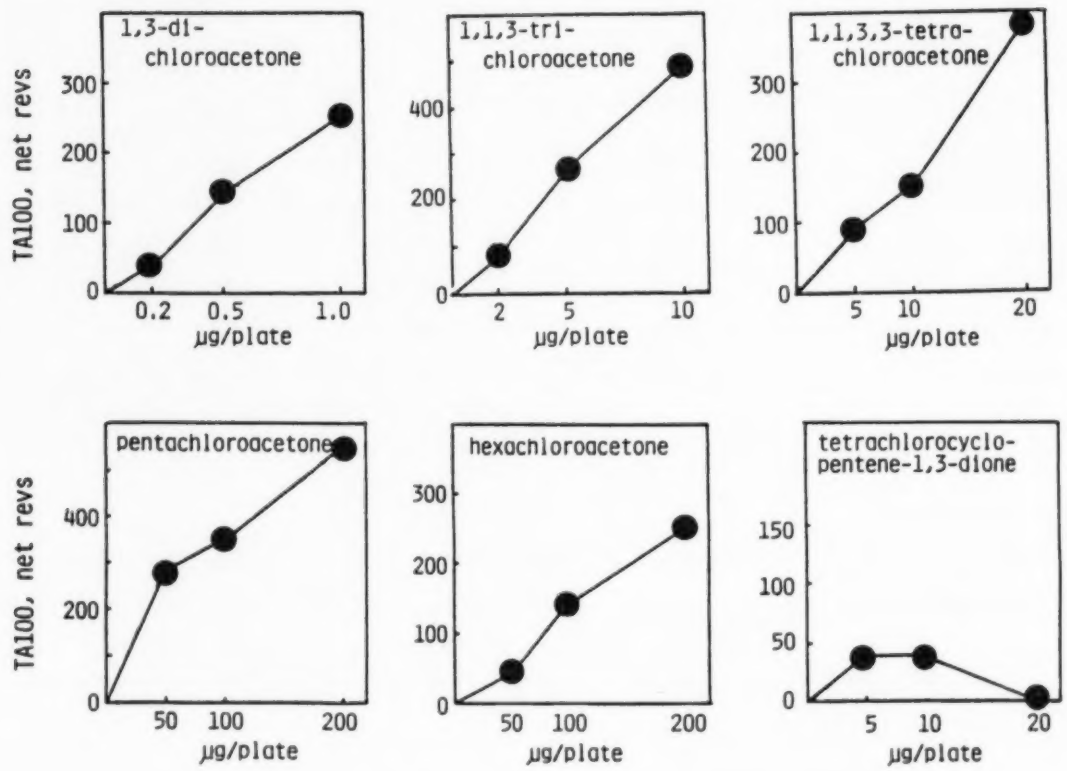

Figure 4: Mutagenic activity, as determined in the Salmonella mutagenicity assay with tester strain TA100 (without S-9), for chlorinated compounds identified from ether extracts of pulp mill effluents. Mutagenicity is expressed in terms of net revertants over a range of 0.2 to 200 ug of compound per test plate.

DISCUSSION

In a previous paper (Kimura et al. 1984), we reported the geographic distribution of chromatophoromas in croaker and pigment cell hyperplasia in sea catfish from Japan. To elucidate the causes of this unique geographic distribution of tumor-bearing fish, several factors, including genetics, viruses, parasites, and chemical contaminants were discussed. No positive results relating to genetic, viral, and parasitic etiology were observed. The data do show that the highest prevalences of skin neoplasms in the two fish species occur in an area (station A) in which a kraft pulp mill discharges effluents into the coastal region. Beginning in 1984, the prevalence of skin neoplasms in croaker at station A showed a marked reduction to approximately 20%. These data may reflect the addition of a new wastewater cleaning system for final effluent of the pulp mill, and the removal of contaminated sediment from the area between 1977 and 1979. Nevertheless, the prevalence of chromatophoromas in croaker at station A still remains the highest among all survey stations (approximately 20%).

In this study, skin neoplasms identical to those observed in wild fish were induced in both fish species by exposure to effluent from a kraft pulp mill. Chromatophoromas in croaker have also been induced by aqueous exposures to nifurpirinol (Kimura et al., 1984); a compound with known mutagenic activity (Nakamura et al., 1982). Therefore, skin pigment cells in these fish species seem to be highly susceptible to the development of neoplastic growth after aqueous exposure to chemicals with mutagenic/carcinogenic activity.

Wastewaters D and E from a kraft pulp mill located in the area where prevalences of skin neoplasms are high, showed direct mutagenic activity in the Ames bacterial mutagenicity assay. By applying mutagenicity-directed fractionation techniques and GC-MS analysis to mutagenic effluent extracts, several substances were identified as mutagens; including 1,3-dichloroacetone, 1,1,3-trichloroacetone, 1,1,3,3-tetrachloroacetone, pentachloroacetone, and hexachloroacetone, as well as methylglyoxal and diacetyl.

These compounds seem mainly to be formed at the bleaching stage in Kraft pulp mills, and appear to be unstable in basic solution (Yamashita et al., 1987). Several chloroacetones have previously been detected in chlorination liquor (McKague et al., 1981) but, in this study, these compounds were also found in the final effluent. The two α-dicarbonyl compounds mentioned above have only recently been identified as occuring in kraft pulp mill effluents (Kinae et al., 1986). Among the above compounds, it was recently reported that 1,3-dichloroacetone (Robinson et al., 1986) and methylglyoxal (Nagao et al., 1986) are carcinogenic in mouse and rat models, respectively. The data showing that the identified compounds contribute only a small proportion to the total mutagenic activity in the effluent extracts indicates that there are other potential mutagens/carcinogens in the effluents that have not yet been identified.

Carcinogenicity testing of other mutagenic compounds using fish species will be essential for elucidating the correlation between environmental contaminants and fish tumors. In Japan, there are many factories which manufacture similar pulp and paper products, such as kraft paper and colour paper. However, in our surveys, fish neoplasms were not observed at high prevalences near these industrial facilities. The kraft pulp mill facility at station A is unique in the following points: a) it is located on the coast facing the Pacific ocean, b) it has discharged waste water to a river estuary for more than 20 years, and c)

in the estuarine area, near the site of effluent discharge, fish congregate to feed upon the rich foods. These environmental conditions may not be matched in other coastal areas.

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The Science of the Total Environment, 94 (1990) 155–167
Elsevier

CARCINOGENIC EFFECTS OF SOME POLYCYCLIC AROMATIC HYDROCARBONS ON THE JAPANESE MEDAKA AND GUPPY IN WATERBORNE EXPOSURES

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ABSTRACT

In this paper, we review studies on the carcinogenic effects of two polycyclic aromatic hydrocarbons (PAHs), benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA), on the Japanese medaka (Oryzias latipes) and the guppy (Poecilia reticulata). Exposure media were prepared by adding PAHs to water, with and without dimethylformamide (DMF) as a carrier, and passing this solution through various sized filters. The low exposure medium was a 0.45 μm filtrate without DMF that contained less than 5 μg L^{-1} PAH. The intermediate medium was a DMF-mediated 0.45 μm filtrate which contained 30-50 μg L^{-1} of PAH. The high medium was a DMF-mediated glass-fibre filtrate which contained 150-250 μg L^{-1} of PAH. Young fish specimens (6-10 d old) were given a 6 h exposure once weekly for 2 to 4 wk. Both BaP and DMBA induced hepatic neoplasms in the two species, with the medaka being more sensitive than the guppy, and DMBA being a stronger carcinogen than BaP. Liver neoplasms almost exclusively developed after exposure to BaP and the neoplasms were limited to the high concentration exposure, whereas DMBA caused a substantial number of extrahepatic neoplasms as well as hepatic ones, especially in the medaka. Furthermore, all three concentrations of DMBA induced hepatic neoplasms in the medaka and all but the low concentration induced neoplasms in the guppy. These studies demonstrate the carcinogenic effects of two waterborne PAHs on two small fish species following brief exposures to very low concentrations, and support the contention that environmental PAHs can contribute to the occurrence of cancer in wild fishes.

INTRODUCTION

Various small fish species are under consideration as carcinogenesis bioassay models because they are economical, develop tumors rapidly, and appear extremely sensitive to some carcinogens (Dawe and Couch, 1984). Compounds that have been shown to be carcinogenic to small fish species include aromatic amines (2-acetylaminofluorene, aminoazotoluene, and 4-dimethylaminoazobenzene), aflatoxins (aflatoxin B_1 and aflatoxin G_1), nitrosamines (diethylnitrosamine, dimethylnitrosamine, nitrosomorpholine, and

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N,N'-dinitrosopiperazine), alkylating agents (methylnitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine) and the azoxy compound methylazoxymethanol acetate (Couch and Harshbarger, 1985; Metcalfe, 1989).

Despite the widespread occurrence of polycyclic aromatic hydrocarbons (PAHs) in aquatic environments (Eisler, 1987), their deleterious effects on aquatic organisms (Neff, 1985), their demonstrated carcinogenicity in laboratory rodents, and their suspected carcinogenicity to humans (Harvey, 1982), few laboratory studies have demonstrated PAH carcinogenicity to fishes. Exposure of rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) to benzo(a)pyrene (BaP) by dietary routes or by intraperitoneal injection (Hendricks et al., 1985), or by injection of embryonated eggs (Black et al., 1985) have shown this chemical to be carcinogenic. Schultz and Schultz (1982) induced hepatocellular neoplasms in two species of small, viviparous fishes of the genus *Poeciliopsis* by repeated short-term exposures to waterborne 7,12-dimethylbenzanthracene (DMBA).

In this paper, we review studies (Hawkins et al., 1988; Hawkins et al., 1989) on the carcinogenic activity of BaP and DMBA to two small fish species, the guppy (*Poecilia reticulata*) and the Japanese medaka (*Oryzias latipes*). We discuss some implications of the waterborne exposure protocols to the bioavailability of PAHs in the environment, the relative sensitivity of small fish to carcinogens, and the utility of these models in experimental carcinogenesis research.

MATERIALS AND METHODS

Test Groups and Preparation of Exposure Media

Test groups included two control groups, an untreated control and a solvent (dimethylformamide, DMF) control, and three PAH exposure groups. The three exposure groups (low, intermediate, and high) were designed to test the carcinogenic response in the guppy and medaka to waterborne PAH in a variety of forms that could simulate the natural environment and achieve a broad range of concentrations as well.

The low exposure medium had the least complex chemical composition and the lowest PAH concentration. This was a true solution of PAH in water that was essentially devoid of extraneous organic and inorganic substances. Since this experiment was not designed to detect low incidences of tumors (less than about 5%), we used PAH solutions at the highest saturation concentrations in this group, which were 1 to 5 $\mu g\ L^{-1}$.

The intermediate exposure medium was only slightly more complex than the low one and incorporated DMF as a solubility enhancer (carrier). In fresh water that is less than pure, natural and anthropogenic substances affect the water's polarity and ionic strength and thus affect the solubility of sparingly soluble organic compounds such as PAHs. This exposure simulated the effect of solubility enhancers that might be present in a natural system. The saturation solubility of PAH (30 to 50 $\mu g\ L^{-1}$) in this medium was chosen as the concentration.

The high dose medium was the most complex because it incorporated a particulate phase of PAH as well as the soluble phase represented by the intermediate medium. This exposure medium reflected a situation in which a PAH is discharged into a natural system at a level exceeding the saturation solubility and which results in the fish being exposed to

both solubilized and particulate PAH. Because of uneven dispersion of PAH particulates, PAH concentrations in this medium varied widely between 150 to 250 $\mu g\ L^{-1}$, and more.

The intermediate and high concentration media were prepared as shown in Figure 1. The low dose medium was prepared by adding granular PAH, either BaP or DMBA (purchased from Aldrich Chemical Company, Milwaukee, Wisconsin), to 3 L of well water to produce a nominal concentration of 10 mg L^{-1}. This suspension was stirred in the dark for 3 or 4 d, filtered through a glass-fiber filter, then filtered again through an 0.45 μm Millipore^R filter. A 200 mL portion of this filtrate was used for chemical analysis and the remaining 2800 mL for exposure to the fish. Exposure media for the intermediate treatment (group 4) and high dose treatment (group 5) were prepared from a stock of PAH dissolved in DMF. In both treatments, PAH dissolved in DMF, as opposed to granular PAH, was added to 3 L of well water to produce a nominal concentration of 10 mg L^{-1}. The intermediate concentration was prepared by passing aqueous PAH with DMF through

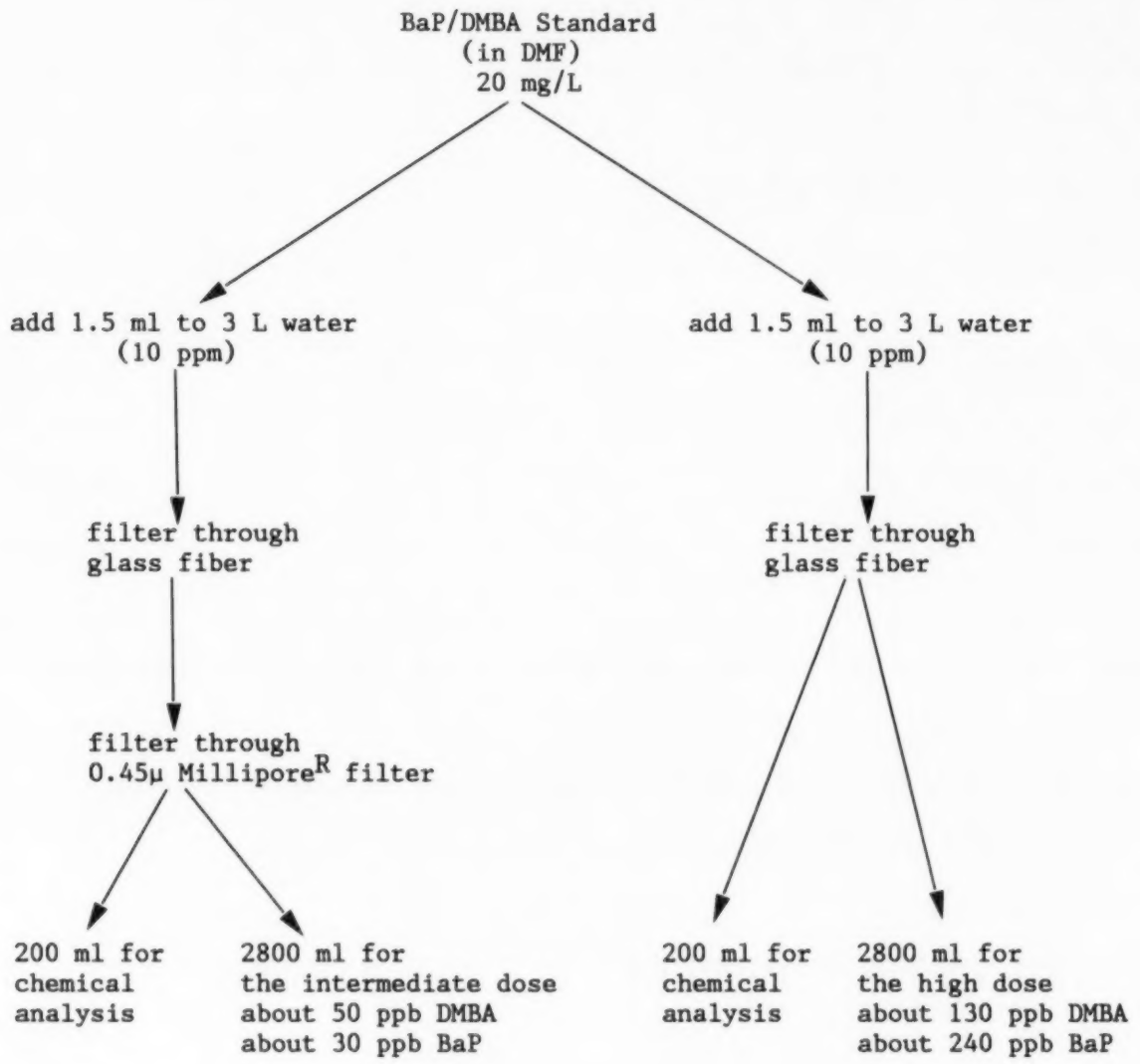

Figure 1. Flow chart for production of media for intermediate and high concentration exposures (Groups 4 and 5).

TABLE 1. Characteristics of exposure groups and concentrations of benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA) in exposure water.

			PAH Concentration[a] ($ug\ L^{-1}$)		
			BaP		DMBA
Exposure Group	Solvent	Filter	Medaka	Guppy	Guppy
Control	None	None	ND[b]	ND	ND
Solvent Control	0.5mg L^{-1} DMF	None	ND	ND	ND
Low Medium	None	0.45um	1;2	4,2	1,3,2,1
Intermediate Medium	0.5mg L^{-1} DMF	0.45um	47,11	32,8	16,30 17,11
High Medium	0.5mg L^{-1} DMF	glass-fibre	269,253	201,217	24,64,28,20[c]

[a] Exposures were for 6 h duration, one week apart; each value represents the mean of duplicate extractions for each exposure period.

[b] Not detectable

[c] Original high dose concentration was toxic, so it was diluted by about 1:10.

a glass-fiber filter, then through a 0.45 μm filter. The high concentration was prepared by passing aqueous PAH with DMF through a glass-fiber filter only. Immediately before fish were added to exposure aquaria, samples of the media were taken for quantitation of PAH concentrations by flame ionization gas chromatography and fluorescence spectrophotometry.

Test Fish and Exposure Methods

All test specimens of Japanese medaka and king cobra guppy were produced from laboratory-reared, disease-free broodstock cultured at the Gulf Coast Research Laboratory. Test specimens were 6 to 10 d old at the time of initial exposure. Exposures were conducted in 1 L glass beakers in a commercial carcinogen glove box (Labconco®) under static conditions, in the dark, at 26°C. Tests began with at least 145 specimens per test group. Initial exposures lasted 6 h, after which the fish were removed to uncontaminated water for 6 d and then exposed one or more additional times in an identical manner on a weekly basis. Fish were not fed during the exposure periods. Following the final exposure, fish were moved to static grow-out aquaria containing uncontaminated water maintained at 26°C. Fish were fed a commercial diet (AquaVet® Stress Formula Flake) supplemented with live brine shrimp daily. Samples for histopathology were collected at 24, 36, and 52 weeks following initial exposure. Moribund specimens were also examined as they were identified.

For histopathological examination, fish were anesthetized with tricaine methanesulfonate (MS-222), examined grossly, and fixed in Lillie's fixative, which contains formalin, formic acid, and picric acid. The abdominal cavities of larger fish were usually opened to facilitate fixation of the viscera. Specimens were dehydrated in alcohol and eventually embedded in paraffin following routine procedures. Specimens were oriented in paraffin blocks so that longitudinal sections through the dorso-ventral plane could be obtained. Each embedded specimen from each test was sectioned and examined histologically in two planes from about mid-sagittal to median. Most sections were stained with hematoxylin and eosin (H&E), but selected ones were stained with periodic acid-Schiff's (PAS). Sections usually contained most major organs, including liver, kidney, gill, intestine, exocrine pancreas, gonad, eye, central nervous system, pseudobranch, thyroid, swimbladder, and heart. Slides were examined without the evaluator knowing any data associated with the specimen, except the specimen number.

RESULTS AND DISCUSSION

The use of a solvent and filters produced widely separated exposure concentrations for BaP and DMBA (Table 1). Moreover, this protocol helped us begin to understand the carcinogenic effects of PAH's presented in soluble versus particulate forms. It was assumed that 0.45 μm filtration, such as that used to prepare the low and intermediate exposure media, partitions soluble from insoluble materials. In that regard, the low and intermediate exposure groups provided the opportunity to study the effects of "true" solutions of PAH at two different concentrations, the higher of which (the intermediate medium) involved an organic carrier. On the other hand, the high medium contained essentially the same concentrations of soluble PAH as the intermediate medium, but contained additional PAH in the form of insoluble particulates. Incidence of hepatic neoplastic lesions in test fishes

TABLE 2. Incidence of hepatocellular neoplastic lesions in medaka and guppy exposed to benzo(a)pyrene.

	Incidence of Lesions					
	24 wk		36 wk		52 wk	
Exposure group	Medaka	Guppy	Medaka	Guppy	Medaka	Guppy
Control*	0/82	0/77	0/89	0/28	-	0/30
Solvent Control	0/75	0/79	0/97	0/70	-	0/39
Low Medium	0/75	0/81	1/94	0/71	-	0/43
Intermediate Medium	0/96	0/84	0/70	0/69	2/11(18%)	0/32
High Medium	8/76 (11%)	7/84 (8%)	26/73 (36%)	13/70 (19%)	4/10 (40%)	8/35 (23%)

* The following extra-hepatocellular neoplastic lesions occurred in control and solvent control medaka specimens: an advanced cystic cholangioma in a 36 wk control; a juvenile rhabdomyosarcoma in a solvent control dying at 16 wk, (see Hawkins et al., 1988) and a cystic cholangioma in a 35 wk solvent control.

(Table 2, Table 3) indicated that the total PAH exposure (soluble plus particulate), as presented to specimens in the high medium, was more carcinogenic than the exposures to solubilized PAH in the low and intermediate concentrations. Furthermore, the enhanced carcinogenicity of particle-containing media is illustrated by the fact that in DMBA-exposed guppies, extrahepatic neoplasms occurred in the high dose exposure group only.

TABLE 3. Incidence of hepatocellular neoplastic lesions in the guppy exposed to 7,12-dimethylbenz(a)anthracene.

	Incidence of Lesions	
Exposure Group	24 wk	37 wk
Control	0/70	1/66 (1.5%)
Solvent Control	0/69	0/60
Low Concentration	0/71	0/50
Intermediate Concentration	7/71 (10%)	12/62 (19%)
High Concentration	35/74 (47%)	23/50 (46%)

Both BaP (Table 2) and DMBA (Table 3) induced hepatocellular neoplasms in the medaka and guppy. After BaP exposure, medaka developed liver tumors sooner and in higher percentages than did the guppy (Hawkins et al., 1988). In both species, DMBA appeared more carcinogenic than BaP, as expected from structure-activity relationships and the results of mammalian bioassays (Yan, 1985). DMBA also induced a considerable number of extrahepatic neoplasms, especially in the medaka (Hawkins, unpublished data). From the standpoint of developing a small fish carcinogenesis model, hepatocellular neoplasms are probably the most significant type of neoplasm because they are the most frequently occurring ones in both laboratory exposures and field surveys (Harshbarger and Clarke, 1989). Although the progression of hepatocellular neoplasms in small fishes is not well understood, our studies on the responses of medaka and guppy to either BaP or DMBA suggest that progression is reflected in three histologic lesions, the altered focus, adenoma, and hepatocellular carcinoma. Whether in relation either to dose or time post-exposure, the lesions appear to arise in that order.

Lesions resembling the altered focus (focus of cellular alteration) occurring in carcinogen-exposed rats (Maronpot et al., 1986), mice (Maronpot et al., 1987), rainbow

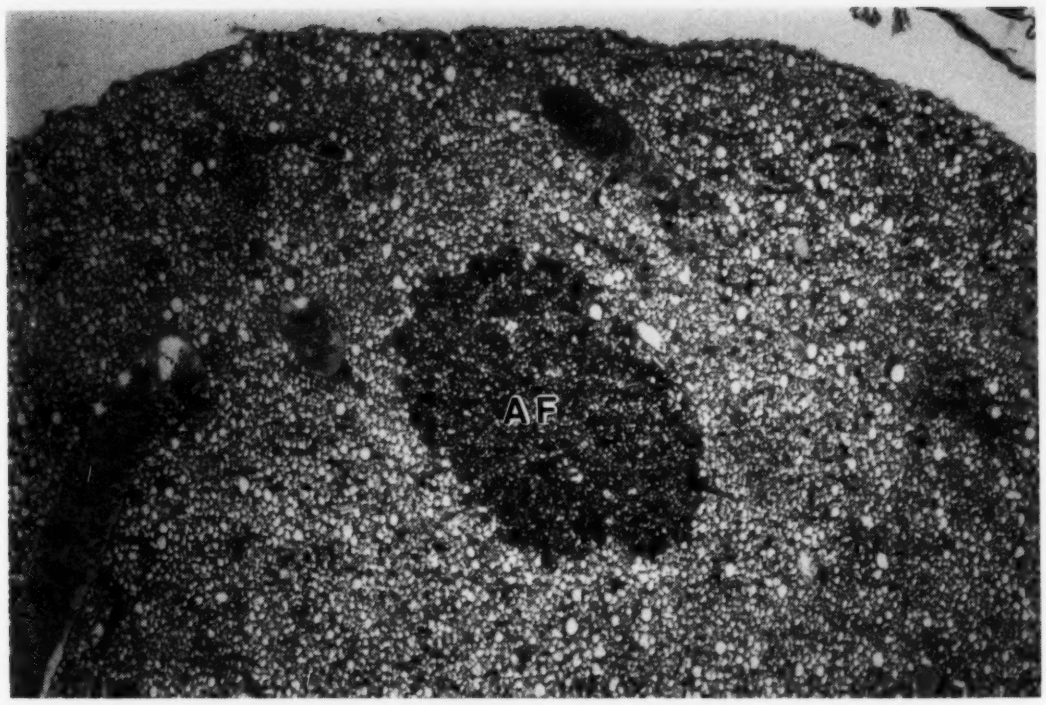

Figure 2. Histologic section of an altered focus (AF) in a guppy exposed to the high BaP concentration and sampled at 36 weeks post-exposure. X 80.

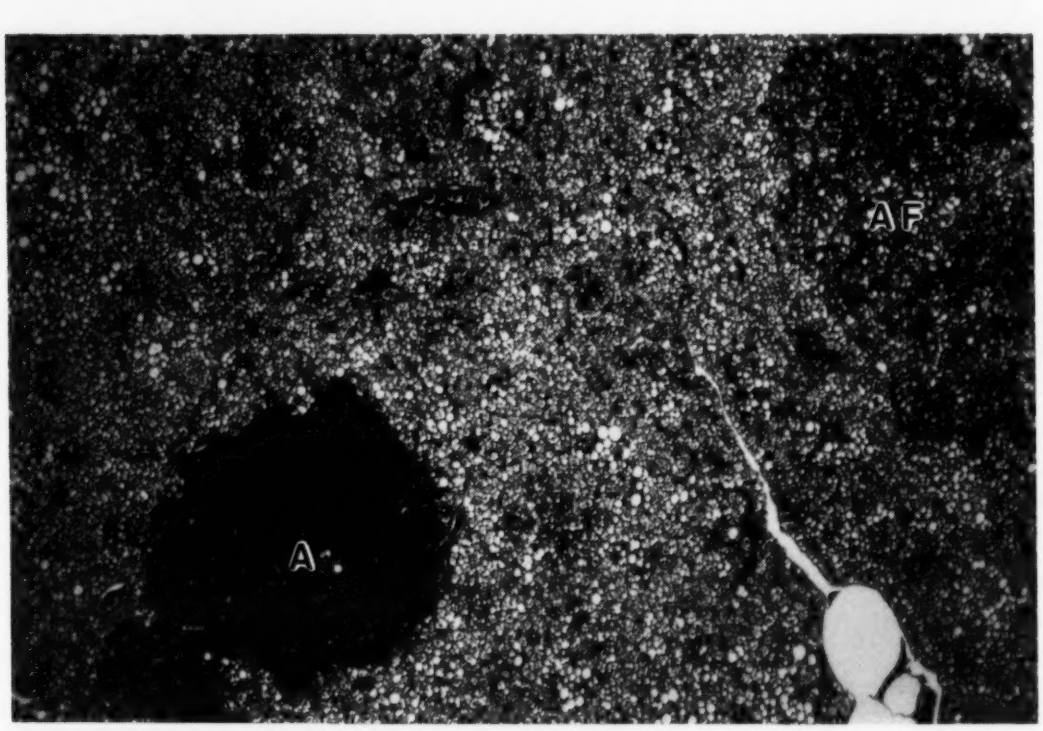

Figure 3. Histologic section showing an altered focus (AF) and an adenoma (A). Note that compared with the altered focus, the adenoma is denser, has a sharper border and is more basophilic. Guppy, high DMBA concentration, 24 weeks post-exposure. X 80.

trout (Hendricks et al., 1984), and some small fish species (Couch and Courtney, 1987; Hinton et al., 1988) are considered preneoplastic lesions that are reversible. Since these lesions in PAH-exposed medaka and guppy occurred, persisted, and appeared to progress and grow in samples taken 24 to 36 wk following brief exposures, they are probably neoplastic. Histologically, medaka and guppy altered foci are usually spherical to oval and consist of hyperbasophilic cells that are continuous with the parenchymal architecture of adjacent normal liver (Figure 2, Figure 3). Figure 3 illustrates our distinction between an altered focus and an adenoma. The adenoma described here is equivalent to the basophilic focus in carcinogen-exposed rainbow trout (Hendricks et al., 1984). Compared with altered foci, adenomas are much more basophilic and have sharper borders. Although adenoma cells are organized like normal liver cells, they are not continuous with the parenchymal architecture of adjacent liver. Hepatocellular carcinomas usually are much larger then either altered foci or adenomas, often are multilobular with irregular outlines, and appear to invade adjacent normal tissue (Figure 4). Although most carcinogen-induced hepatocellular lesions fit into one of those three categories, what appear to be intermediate stages exist between the altered focus and adenoma, and hepatocellular carcinoma appears to develop from the adenoma. Occasionally, some lesions such as the one illustrated in Figure 5 do not fit the above categories. That example appeared to be proliferative, to consist of glycogen-loaded cells, and to grow centrifugally, compressing adjacent parenchymal cells.

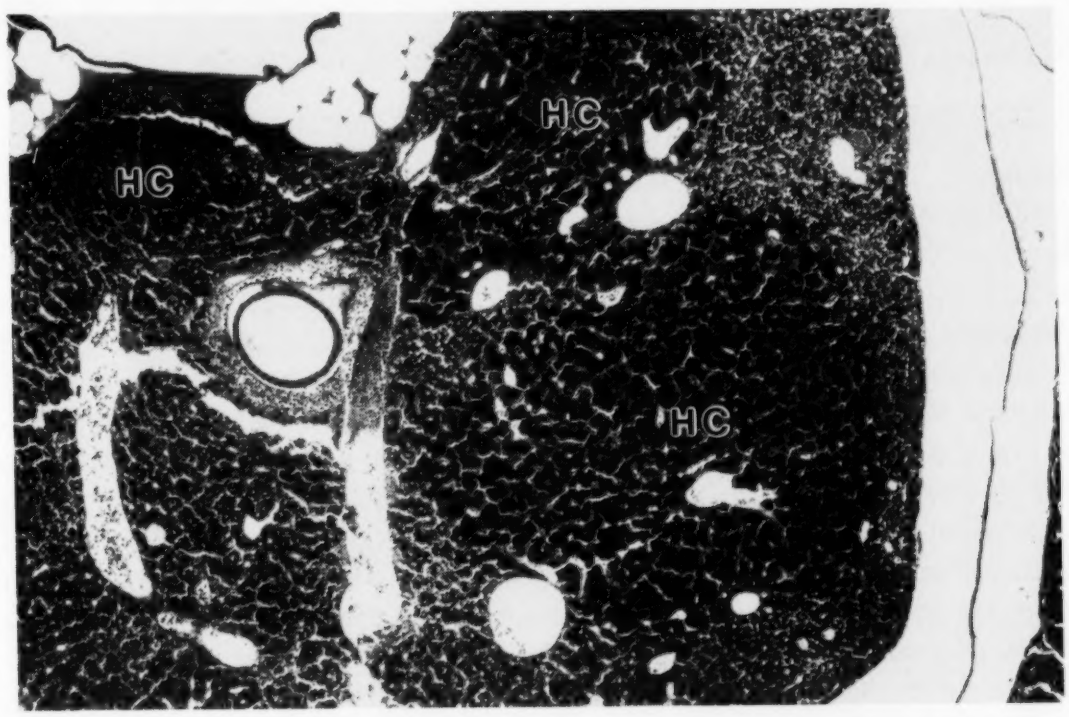

Figure 4. Histologic section of the liver of a medaka from the high BaP concentration, 36 weeks post-exposure showing three lobes of a hepatocellular carcinoma (HC) that has invaded most of the liver tissue. X 80.

Figure 5. Histologic section of an unclassified lesion (L) that appears to be proliferative and to compress surrounding normal liver (arrowheads). Medaka, high BaP concentration, 36 weeks post-exposure. X 30.

Studies on the carcinogenicity of BaP and DMBA to small fish species suggest that fishes might be good models for examining the carcinogenicity of environmental PAHs that occur either in sediment or water. In the field, fishes are probably exposed to PAHs through a variety of pathways that include direct exposure from the water, contact from the water and the sediment, and dietary uptake through consumption of PAH-contaminated organisms and associated sediment. Accumulation of PAHs from contaminated food is usually less predictable than PAH uptake from solution and is highly species-dependent, both for the food organism and the consumer (Neff, 1985). We have directed little attention to the routes by which medaka and guppy are exposed to PAHs. We assume that soluble PAH uptake takes place across superficially located epithelia such as the gill, although the efficiency of transport of high molecular-weight PAHs across the gill epithelium may be low. Perhaps the enhanced carcinogenicity of media containing PAH particulates results from particles that reside directly on gill membranes for some period of time before being flushed away by water circulating across the gills. During that time, solubilization of particles adjacent to active gill membranes could promote PAH incorporation into the fish. The PAHs are likely then to be transported to the liver where they are metabolized by the microsomal mixed function oxidase (MFO) system. We have shown that 3-methyl-cholanthrene induces hepatic MFOs in medaka and guppy, and that the medaka is capable of producing BaP dihydrodiol epoxides which are the ultimate carcinogens resulting from the metabolism of BaP (James et al., 1988).

Cancer epizootics in fishes in Puget Sound and tributaries of the Great Lakes have occurred mainly in bottom-dwelling species living near PAH-contaminated sediments

(Harshbarger and Clark, this volume). In one PAH-impacted area of the Puget Sound, up to 54% of a sample of 151 English sole had some form of hepatic neoplasm or related lesion (Myers et al., 1987). Prevalences of hepatic neoplastic lesions have been correlated with aromatic hydrocarbon levels in marine sediments near where the fish were collected (Malins et al., 1985), and with levels of metabolites of aromatic compounds in the bile (Krahn et al., 1986). Since English sole are capable of biotransforming BaP to a carcinogenic dihydrodiol intermediate that covalently binds with hepatic DNA (Varanasi et al., 1987), aromatic hydrocarbon exposure seems a likely cause of the neoplasms in these fish. Because the English sole is not presently amenable to laboratory culture from the egg to adult stage, attention must be turned toward other fish models for experimental evidence of PAH carcinogenicity. Hendricks et al. (1985) showed that rainbow trout either fed BaP in their diets or injected intraperitoneally with BaP, developed liver tumors. Schultz and Schultz (1982) induced hepatic neoplasms in two species of *Poeciliopsis* with waterborne exposures of DMBA, and demonstrated that DMBA-induced tumors were transplantable (Schultz and Schultz, 1985). Hepatic lesions in English sole from Puget Sound are far more complicated (Myers et al., this volume) than those we see in PAH-exposed medaka and guppies, possibly because of exposures to complex mixtures which contain tumor initiators and promotors, as well as hepatotoxic compounds. Nevertheless, the basic pattern of tumor development beginning with the altered focus, followed by the adenoma, and finally ending with the hepatocellular carcinoma that occurs in the small fishes can also be seen in the English sole. At the present time, comparisons of field observations with laboratory experiments in regard to lengths of exposure, dosages, and routes of exposure have not been attempted. Our studies showed that DMBA and BaP exposures at $\mu g\ L^{-1}$ concentrations lasting a total of 12 to 24 h induced tumors in high percentages in exposed guppies and medaka within six mo. In comparison, PAH concentrations related to cancer in indigenous fish populations are often reported in sediment at $\mu g\ g^{-1}$ levels (Black, 1983; Baumann and Harshbarger, 1985; Malins et al., 1985; Metcalfe et al., this volume), and exposure times could last several years or for the lifetime of the fish.

ACKNOWLEDGEMENTS

These studies were supported in part by a contract with the American Petroleum Institute.

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3 R. D. Marshall and A. Beuberger, in A. Gottschalk (Ed.)., Glycoproteins, Vol. 5, Part A, Elsevier, Amsterdam, 2nd edn., 1972, Ch. 3, p. 251.

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THE SCIENCE OF THE TOTAL ENVIRONMENT

Vol. 94 Nos 1 + 2 **Contents** **May 1st, 1990**

(Abstracted/Indexed in: Biol. Abstr., Chem. Abstr., Curr. Contents AB & ES, Environ. Period. Bibliogr., Excerpta Med., Geo Abstr., Oceanogr. Lit. Rev., PASCAL/CNRS, Selected Water Resour. Abstr.)

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For Contents, see page vii

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0048-9697/90/$03.50

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